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FOREWORD

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**GEORGETOWN INSTITUTE
FOR COGNITIVE AND
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SCIENCES**

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INTRODUCTION

The Georgetown Institute for Cognitive and Computational Sciences (GICCS) is a neuroscience research institute whose mission is to understand higher cognitive function through interactive collaborative efforts among scientists using multidisciplinary investigative strategies. Its major focus areas are: brain injury and plasticity; higher auditory processing and language; computational neuroscience; and drug discovery.

GICCS faculty are working to elucidate the complex mechanisms of higher auditory processing and language. Research in humans uses functional brain imaging and cognitive psychology to examine how the human brain deals with complex sounds, particularly those relating to speech. These studies address not only normal language processing but also disorders of speech/language, including developmental and acquired dyslexias. One goal is to develop treatments for these disorders through specialized training.

Understanding and modifying brain plasticity represents another major research area. Investigators use tools from cellular/molecular neurobiology and from systems neuroscience to study plasticity after acute or chronic brain injury, as well as relating to development. This includes design of novel pharmacological strategies to limit brain damage and to enhance cognitive function after injury or neurodegeneration. Brain magnetic resonance techniques (including functional imaging) are also used, employing a high field (7T) animal research magnet as well as a human 1.5T magnet, to clarify mechanisms of tissue damage, plasticity and the response to targeted treatments. In addition to advanced brain imaging, computational neuroscience is an important experimental area that serves to integrate these multidisciplinary research efforts. Molecular modeling approaches are also used to study protein folding and to enhance drug discovery.

Together, the goal of this diverse but complementary research team is to better understand cognitive processes in order to address important clinical problems including deafness, language disorders, traumatic and ischemic brain injury, and Alzheimer's Disease.

Faculty includes 3 professors, 1 associate professor, 9 assistant professors, and 4 research associates. A total of 34 postdoctoral fellows and 19 research technicians support the faculty research programs. Faculty are divided into sections: cognitive neuroscience (human); cognitive neuroscience (animal); computational neuroscience; drug discovery and design; and molecular neurobiology and plasticity. There are numerous collaborative connections within and across these sections. For example, all members of the human cognitive group and several members of the animal cognitive group share a common interest in the use of magnetic resonance imaging technology to address fundamental questions. Magnetic resonance technology also links the animal cognitive group with the group in molecular biology and plasticity. The drug development and design group has extensive collaborations both within the Institute as well as involving other departments at the Medical Center. Research developments during the past year are divided into 5 parts, one relating to each institute section.

HUMAN COGNITIVE NEUROSCIENCE: Dr. Eden uses functional neuroimaging to study the pathophysiology of developmental dyslexia. Dr. Friedman examines the neuropsychology of language. Dr. Liu's research focuses on both the development and application of functional Magnetic Resonance Imaging (fMRI) of human brain. Dr. Ullman investigates neural bases of language and memory. In addition, Dr. Rauschecker conducts research projects in Human Cognitive Neuroscience using fMRI.

GUINEVERE EDEN, D.PHIL.

Guinevere Eden's laboratory uses behavioral testing and functional magnetic resonance imaging (fMRI) to characterize sensory and cognitive processing in individuals with and without developmental dyslexia. Developmental dyslexia is a common developmental disorder (observed in 5-10% of the population) in which individuals fail to acquire normal reading skills. It has been shown to result from microscopic anomalies at the neuronal level, such as hetero-topias in perisylvian regions (Galaburda, et al., 1989) and maldevelopment in the magnocellular subdivisions of the visual and auditory pathways (Livingstone et al., 1991; Jenner et al., 1999). Neuronal injury is thought to occur in the prenatal period, resulting in changes in neuronal migration. These injuries are predominantly triggered by factors that have a genetic origin (Olson et al 1991). The behavioral manifestations of dyslexia are complex, involving abnormalities in both language and sensory perception. The neuroanatomical findings have been related to the deficits observed in the perception of fast-changing stimuli (Tallal, et al., 1993) and it has been suggested that the language and perceptual visual, auditory and motor problems of developmental dyslexics may stem from some abnormality in the 'timing' mechanisms necessary for normal language processing (Tallal, et al., 1991). The identification of areas of cortical maldevelopment implying early brain injury in developmental dyslexia have triggered brain-based research utilizing functional brain imaging. Specifically, in dyslexia the absence of the ordinary pattern of leftward asymmetry of the planum temporale and the perisylvian cortex and foci of ectopic neurons in the molecular layers have been reported. Some brains showed multiple foci of glial scarring, leading to a proposal of ischaemic injury to the developing cerebral cortex produced by autoimmune damaged vessel walls (Sherman et al., 1989). How these cortical injuries, the scars and malformations, are related to cognitive and sensory processing deficits can be elucidated with functional brain imaging. These studies include examinations of rapid visual, auditory and motor processing as well as phonological awareness. Although these processes have been previously shown to be impaired in dyslexics, the nature of their shared contribution and questions concerning a common neocortical etiology are being investigated for the first time.

PROJECT 1: FMRI OF PHONOLOGICAL AND SENSORY PROCESSING IN DYSLLEXIA

1a: Developmental Dyslexia and Phonological Awareness

Introduction: The most widely accepted current explanation for dyslexic's reading difficulties involves abnormal phonological processing. The term "phonological awareness" has been used as an umbrella term for the skill of manipulation and segmentation of the constituent sounds of words (Stanovich, 1988). The neuronal mechanisms that subserve the type of rapid phonological retrieval and phonological segmentation impaired in dyslexia are not yet clearly defined. Some of the differences observed in dyslexics during previous functional neuroimaging studies are difficult to interpret due to

our incomplete understanding of "normal" language processing. With advancements in neuroimaging technology, it is now possible to study an individual subject's performance during a wider variety of tasks than was previously possible.

Methods: We utilized the BIG technique (Eden et al, 1999) to investigate phonological awareness skills in 16 adults. The tasks utilized required an awareness of the sound structure of language and an ability to perceive and manipulate both auditory phonemes and written graphemes. The following four tasks were employed, using both written and auditory presentations of single words with equal levels of difficulty, in order to study phonological processing: (1) elision, (2) segmentation, (3) rhyme judgement, and (4) semantic category judgement. Data were acquired utilizing a multislice echo-planar image (EPI) technique (40 msec TE, 13 sec TR, 64x64 matrix, 50 axial slices and 3 mm cubic voxels). MEDx was used for functional image analysis and visualization. Each EPI time series was corrected for head motion, global intensity variation and local (temporal) intensity variations. Statistical maps were generated by contrasting task and control conditions for each subject. For neuroanatomical localization the functional data were registered with a high-resolution anatomical scan.

Results: The left occipito-temporal and inferior parietal cortex was activated uniquely and significantly during all measures of PA (See Figure 1 for results). Most importantly, this functional specificity was observed during phonological (but not semantic) processing in response to spoken as well as written words, supporting a specific role of this extrastriate and inferior parietal area in a skill that is fundamental to reading. These results support the notion that these areas are specific to phonological processing but are independent of whether the linguistic stimuli that require phonological manipulation are coded visually or orally. Currently we are investigating task-related brain activity differences during these tasks in individuals with dyslexia.

1b: Developmental Dyslexia and the Visual System

Introduction: Functional neuroimaging utilizing positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have identified a specific motion sensitive area, V5/MT, dominated by input from the magnocellular stream. Behaviorally these channels can be distinguished by their spatial frequency preference, their temporal properties and their contrast sensitivity. Both contrast sensitivity and visible persistence has been shown to be varied in reading disabled individuals, indicating that these individuals have disturbances in the magnocellular or transient system, which mediates global form, movement, and temporal resolution (Lovegrove, 1993; Lovegrove & Brown, 1978; Lovegrove et al., 1980; Eden et al, 94; Eden et al 95). We investigated such a motion processing deficit in the visual system of dyslexics (Eden et al., 1996) and found that passive perception of visual motion in dyslexics failed to produce any detectable task-related functional activation in area V5/MT (part of the magnocellular system). In contrast, all control subjects had a robust response in the same region. We concluded that dyslexics suffer from abnormalities of the fast visual processing pathway (magnocellular), whilst the slower form processing system (parvocellular) was unaffected. This profound physiological abnormality was accompanied by a relatively subtle behavioral deficit in visual motion detection. This finding of an V5/MT deficiency in dyslexia has recently been replicated in an fMRI study that showed activity in this area to be directly correlated with reading skill (Demb, Boynton & Heeger, 1997, Eden and Zeffiro, 1998). Using radial motion, we present a study of the entire brain to investigate radial visual motion processing in individuals with and without dyslexia.

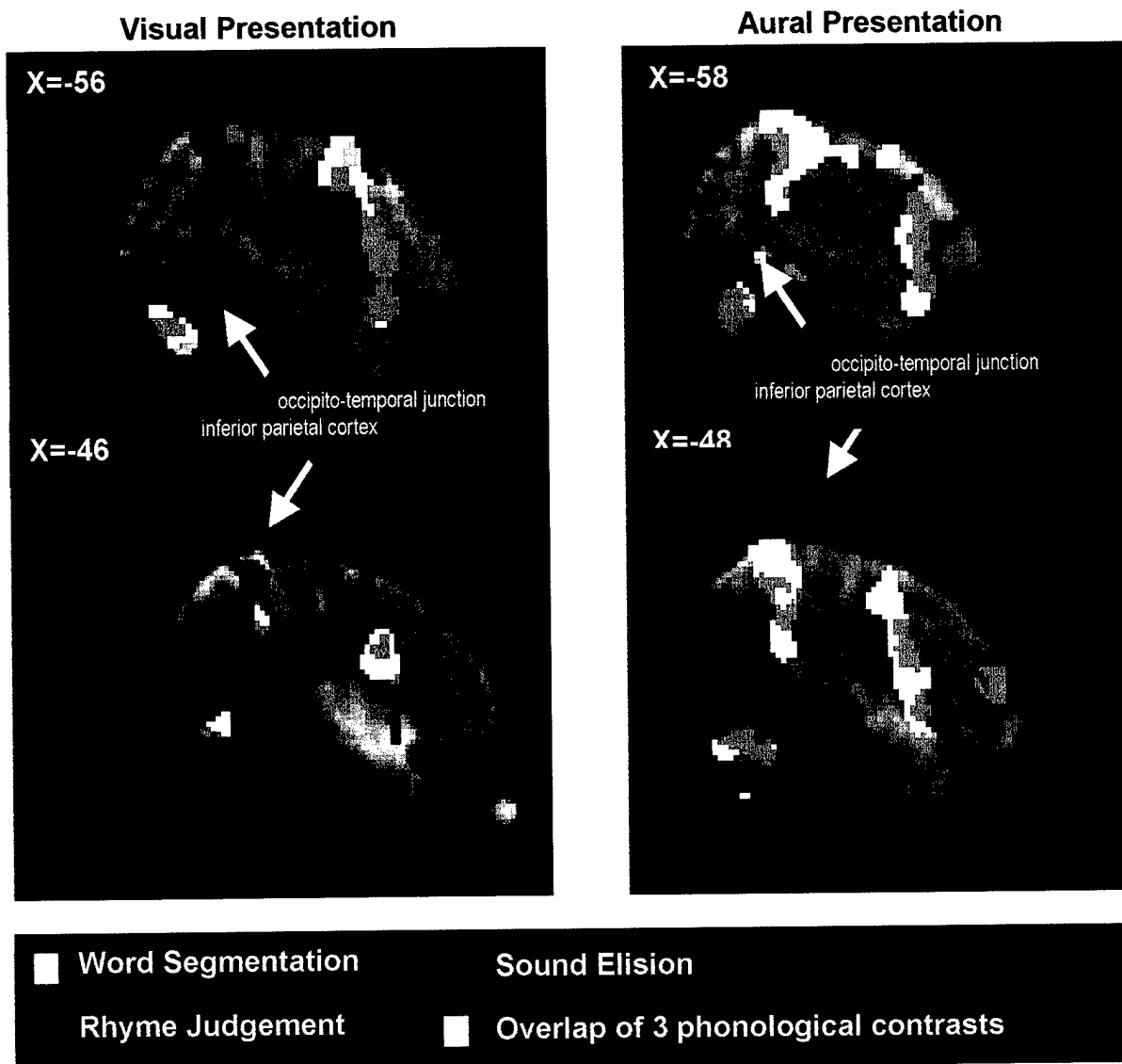


Figure 1: fMRI Task-related Signal Change Associated with Three Phonological Awareness Tasks

Methods: Fifteen dyslexic and 22 control subjects viewed 3 different visual stimuli: (1) FIXATION: a light gray cross-hair centered on a dark gray background, (2) STATIC: 300 randomly arranged light gray dots on a dark gray background, and (3) RADIAL MOTION: 300 light gray dots radiating centrifugally from the central fixation point. The luminance was the same in all conditions and the stimuli subtended 52 degrees of visual angle. The 24 second epochs were presented in the following order: fixation, static, fixation, motion, etc. for a total of 32 epochs per run. Data were acquired utilizing a multislice echo-planar image (EPI) technique (40 msec TE, 6 sec TR, 64x64 matrix, 50 axial slices and 3 mm cubic voxels). MEDx was used for functional image analysis and visualization. Each EPI time series was corrected for head motion, global intensity variations and local (temporal) intensity variations. Statistical maps were generated by contrasting task and control conditions for each subject. For neuroanatomical localization the functional data were registered with a high-resolution anatomical scan.

Results: In each subject the RADIAL MOTION versus STATIC contrast revealed bilateral task-related increases in striate and extrastriate visual cortex (including area V5/MT), anterior cingulate gyrus, inferior frontal gyrus, anterior superior temporal gyrus, posterior parietal cortex and posterior cerebellum (Joseph et al., 1998) in the non-dyslexic group. Differences in area V5/MT were observed between the dyslexic and non-dyslexic groups, replicating our previous study.

1c: Co-localization of visual motion and phonological processing in extrastriate cortex

Introduction: Individuals with reading problems have deficits with both visual motion and phonological processing as shown by behavioral and functional neuroimaging studies. To investigate the possibility of a common neuronal substrate for these apparently different tasks, we conducted two separate fMRI experiments in eleven normal adults.

Methods: First, participants passively viewed radial visual motion alternating with static dots and a fixation cross. The statistical contrast of visual motion versus static dots revealed that human area V5/MT was strongly activated in all subjects. Secondly, participants performed three different phonological tasks with visually and auditorally presented words: sound deletion, word segmentation and rhyme judgement. Statistical contrasts of each of these tasks with word reading or word repetition were performed (see above for details on data acquisition and analysis).

Results: The three phonological tasks activated a network of regions, including extrastriate, inferior parietal and inferior frontal cortices. The extrastriate region was most selective for phonological processing. Group and single subject analyses revealed close spatial proximity in extrastriate cortex for task-related signal changes associated with both phonological and visual motion processing. This striking co-localization may offer an explanation for the visual motion and phonological deficits observed in dyslexia (see Figure 2).

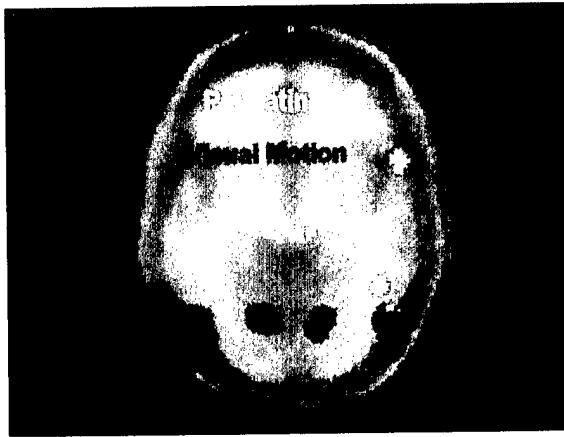


Figure 2: fMRI Task-related Signal Change Associated with Phonological Awareness and Visual Motion Processing Tasks (right side of image corresponds to left side of brain)

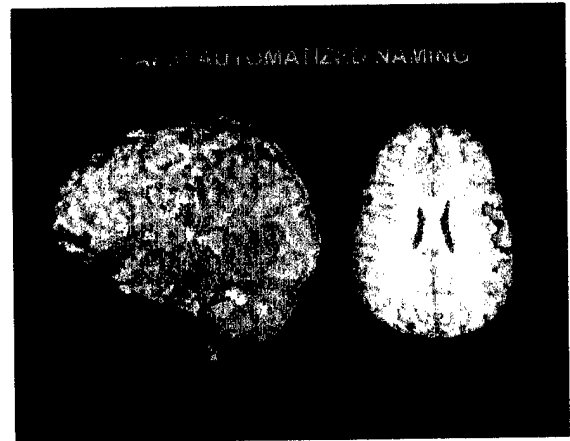


Figure 3: fMRI Task-related Signal Change Associated with Rapid Automatized Naming

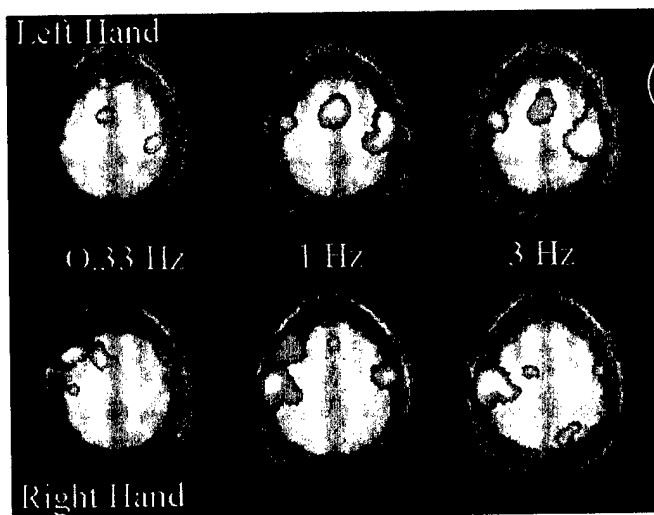


Figure 4: Foci of neuronal activity in the primary motor cortex for each of six conditions. From left to right, the frequency increases from 0.33 Hz to 3 Hz. Axial sections (top) depict task-related signal change when the subject tapped with the left thumb and (bottom) when they tapped with the right thumb.

PROJECT 2: NEUROPHYSIOLOGICAL CORRELATES OF RAPID NAMING IN DYSLEXIA

Introduction: Rapid automatized naming (RAN) is widely used to differentiate individuals with dyslexia, yet the cognitive processing sub-components of rapid naming are not well understood. The present project examines three cognitive aspects of rapid naming – phonological, lexical/semantic and automatized processing – and uses functional magnetic resonance imaging (fMRI) to elucidate the neural systems involved in these component processes. For example, RAN difficulties observed in dyslexia may not be restricted to phonological problems. RAN impairment may also reflect problems with the automaticity of phonological decoding. In dyslexic readers, the conversion from visually presented letters or words to sounds is not as automatic as it is in normal readers. This impairment may be specific to verbal materials or it may reflect a more general learning problem. The present study uses implicit temporal sequence learning in the context of rapid naming to assess whether dyslexics are impaired in implicit learning, or whether their RAN deficit can be explained by a specific problem with automatizing verbal material.

Methods: We utilized the BIG technique (Eden et. al, 1999) to investigate rapid naming in 12 adults (see above for data acquisition and analysis procedures). Subjects named common colors and objects, at 3 different rates. Parametric variation was used to identify the areas that are rate-modulated during this task. The tasks utilized required rapid phonological retrieval and individuals with dyslexia perform this task at a slower rate. It is therefore important to understand the relationship between task-related signal change and rate related changes in this task.

Results: Data has been acquired in 12 individuals with good reading and rapid naming skills and is currently undergoing analysis. Preliminary data from an individual with normal reading skills are shown in Figure 3. The neuroimaging findings obtained in this study will help our understanding of the functional organization of neural systems involved in impaired and normal rapid naming. These systems are likely to be shared with those areas subserving phonological processing skills described above and known to be altered in individuals with developmental dyslexia.

PROJECT 3: FMRI STUDIES OF MOTOR SKILLS IN DYSLEXIA

Introduction: In this study, functional neuroimaging is being used to investigate the physiological basis of motor coordination. Dyslexic individuals show impoverished performance during bimanual finger tapping at high rates. At least three theories have been developed to explain the impaired sensorimotor performance observed in dyslexia and are based on observation of abnormal cell migration or neuronal development in these areas. One theory posits the existence of disrupted interhemispheric communication, resulting in poor performance on sensorimotor coordination tasks. In this account, poor performance on tasks involving precise intermanual coordination results from failure to coordinate the activities of the cortical motor areas of the two hemispheres. A second theory suggests that there is a generalized left hemisphere dysfunction affecting language processing and fine motor control mechanisms. Dysfunction of the cortical motor areas in the left hemisphere may impair fine motor movements using both contralateral and ipsilateral hands. The third hypothesis places the neuroanatomical locus of the dysfunction in the cerebellum, explaining deficits in both intermanual coordination and motor learning. In the context of these three theories, this series of experiments is

designed to explore the breakdown of this sensorimotor integration system in individuals with developmental dyslexia. Between-group comparisons will be conducted to determine whether the patterns of neuronal activation identified in dyslexic individuals differ from those exhibited by normal volunteers while performing the unimanual and bimanual motor tapping tasks at different rates.

Methods: Task-related signal changes were measured using fMRI while subjects performed a tapping task in which they respond to a visually-presented stimulus. Response time and response accuracy were recorded. Both tapping frequency and method of response was varied. Tapping frequencies of 0.33 Hz, 1 Hz and 3 Hz were used based on previous behavioral and neuroimaging experiments. The method of response was as follows: (1) left hand alone, (2) right hand alone, (3) both hands synchronously, and (4) both hands asynchronously.

Results: To date 12 subjects have been scanned and data analysis is ongoing. Preliminary analysis demonstrates parametric variation of task-related signal change in primary motor cortex and cerebellum. The results are shown in Figure 4.

SUMMARY

Integration of the resulting behavioral, anatomical and physiological information into structure/function correlations is a principal goal of our research program. Using these techniques we will examine the degree to which these physiological changes correlate with the observed behavioral deficits. Comparison of results across modalities will reveal to what degree dyslexics exhibit functional deficits common to vision, audition, and motor control and may suggest the neuroanatomical localization of a common neural substrate. The extrastriate area at the occipital-temporal junction and the inferior parietal cortex in the left hemisphere are likely to play an important role. The results of these experiments will provide new information concerning the neural substrates responsible for the visual, auditory and phonological abnormalities characteristic of developmental dyslexia. Moreover, the laboratory is interested in the development of new diagnostic tools that may allow earlier and more accurate identification of individuals with developmental language disorders and in the application of brain imaging in the assessment of intervention.

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DR. RHONDA B. FRIEDMAN, Ph.D.

Dr. Friedman's research encompasses three major projects: (1) Cognitively-based treatments of acquired dyslexias; (2) Written language processing in Alzheimer's Disease and related disorders; and (3) Evaluating cognitive neuropsychological models of language recovery with fMRI.

PROJECT 1: COGNITIVELY-BASED TREATMENTS OF ACQUIRED DYSLEXIAS

The purpose of this project is the development of a set of therapy programs that are shown to be effective in the treatment of acquired disorders of reading (acquired dyslexias, also known as alexias). This goal is achieved through the development, implementation, and evaluation of several experimental therapies, each targeted for a specific type of reading deficit, based upon a cognitive neuropsychological model of reading. The data obtained from this study are also used to improve our models of normal reading, which may lead to more effective methods of teaching reading to both normal and developmentally dyslexic children.

Patients with acquired reading disorders following stroke are referred to our project by neurologists or speech pathologists for further evaluation. The patient's reading and other cognitive skills are assessed using a battery of screening tests that we have developed over the past several years. Based upon the results of these tests, the patient's alexic disorder is characterized. Patients whose deficits are among those that are the focus of this project are assigned to one of the treatment programs devised specifically for that type of deficit. For many, the question of bypassing an impaired system vs. remediating the disturbance is addressed. When feasible, factors that might predict the success of a particular treatment for a particular patient are examined. The overall structure of the study consisted of single case studies, replicated over several patients, each employing a multiple baseline design.

The following results have been obtained over the past year.

STUDY 1. PURE ALEXIA SIGHT WORD TREATMENT

The initial phase of our original hybrid treatment consisted of training rapid recognition of a set of the most frequently occurring words. We previously reported that patient FT successfully learned to read these words. We are currently training another patient, BS, in this paradigm. In addition we are analyzing his performance on these and other stimuli both before and after treatment (see fMRI section for details).

For 20 sessions we initiated training on the first of 6 sets of 40 words each. However, due to time constraints, we decided to focus treatment solely on the words being assessed by fMRI. A single training set, composed of the 68 4-letter words contained in the initial 6 sets, was created. After 12 training sessions it appeared that this set contained too many words to be learned simultaneously. The set was then divided into 3 sets of approximately the same number of words, and baseline performance was assessed. After 6 training sessions to date, BS's recognition of the first set of these words is improving (See Table 1).

STIMULUS	BEFORE TRAINING	TRAINING TO DATE
Set 1 words	28%	65%
Set 2 words	27%	Not yet trained
Set 3 words	30%	Not yet trained

TABLE 1. BS's accuracy rapidly recognizing words before and after training.

STUDY 2: PHONOLOGICAL/DEEP ALEXIA

Patients with this type of alexia cannot read via a system of assembled phonology (letter - sound conversion). Thus unfamiliar words and pronounceable nonwords are read poorly relative to real familiar words.

1. Semantic Mediation Treatment. In contrast to our Phonological Neighborhood treatment which concentrated on "restoring" the damaged route to reading, this therapy takes the "re-organization" approach. It attempts to use the semantic route to circumvent the problems in the phonological reading route. This treatment pairs difficult words low in semantic value (functors and verbs), which are difficult for these patients to read, with homophones (words that are pronounced the same) or near homophones (e.g. in and inn, me and meat) that have high semantic value. The word pairs are trained via flashcards; the front contains the written target word and the back contains the written homophone along with its picture. Two sets of targets were trained sequentially.

New subject ST improved his reading of target words (see table 2), thereby replicating previously reported results. However, spontaneous recovery could not be ruled out, as a set of untrained words also improved. To date, 3 patients have improved their reading of the target words, supporting the hypothesis that semantic route reading may be invoked to re-organize impaired phonologic route reading. Furthermore, previous data suggested that the use of homophones is critical to the success of this treatment.

STIMULUS	BEFORE TRAINING	AFTER TRAINING
Set 1 words	16%	97%
Set 2 words	13%	100%

TABLE 2. ST's reading accuracy of target words before and after training.

The semantic mediation treatment was successful in improving the subjects' reading accuracy, but response time remained slow. In order to improve functional reading, treatment focusing on speed and sentence level reading was created. High frequency nouns and 50 of the trained words were used to compose sentences. Prior to sentence training, the 50 trained words were trained in a speeded paradigm. The remaining 30 words that were trained in the original paradigm were not trained in this speeded paradigm, but were regularly probed to assess generalization. Words were presented one at a time on a computer screen. The subject was instructed to read the word as quickly and accurately as possible, and his/her response time was recorded. As feedback, the subject was told his/her average response time. Treatment continued until the subject reached asymptote.

HN improved both her speed and accuracy of reading the speeded and non-speeded words (See table 3). In addition, this effect generalized to improved accuracy reading sentences composed of speeded words (see table 4, column 2).

STIMULUS	BEFORE TRAINING	AFTER TRAINING
Speeded words		
Response time	24 seconds	7 seconds
Accuracy	65%	95%
Non-speeded words		
Response time	23 seconds	6 seconds
Accuracy	71%	85%

TABLE 3. HN's reading speed and accuracy of speeded and non-speeded words before and after training.

Subject DN also participated in this treatment. He demonstrated some improvement initially, but performance then declined. This may be attributable to his declining medical status, as he was observed to be more fatigued and confused than he had been earlier in the study. Subject DN was withdrawn from the study at this point.

Despite having learned to read a set of words quickly and accurately, HN continued to read sentences composed of those words quite poorly. The next phase of treatment consisted of training HN to read these sentences by focusing on one word at a time. HN improved her reading accuracy not only of trained sentences, but of untrained sentences that were regularly probed and untrained sentences that were probed only before and after each treatment phase as well (see table 4).

STIMULUS	BEFORE SPEEDED WORD TRAINING	AFTER SPEEDED WORD TRAINING	AFTER SENTENCE TRAINING
Training Sentences	2%	15%	98%
Untrained Sentences	2%	18%	89%
Pre/Post Sentences	5%	18%	55%

TABLE 4. HN's sentence reading accuracy before and after speeded training and after sentence training. Sentences in all three conditions were composed only of words that had been trained for speeded reading.

PROJECT 2: WRITTEN LANGUAGE PROCESSING IN ALZHEIMER'S DISEASE

This study examined the nature of the decline in spelling abilities in patients with probable AD. Twenty-three patients with probable AD and 27 normal controls were asked to write different kinds of real words and pronounceable pseudowords (PWs) to dictation. The real words included those with regular spellings (e.g. hand), those with exceptional spellings (e.g. choir) and those with ambiguous spellings (e.g. heat). PW types were similar to the word types. Words and PWs were blocked, and were presented auditorily. Subjects wrote their responses.

Results indicate only one effect that differed between groups, that of regularity; AD patients showed a small but significantly greater difference between real words and exception words than did the normal controls.

AD patients performed more poorly overall, but error types were not qualitatively different from controls. In addition, when spelling PWs with several different acceptable pronunciations, AD patients showed preferences similar to controls. Dementia severity was related to overall accuracy, but not to pattern of spelling responses. The results suggest that decline in spelling accuracy in patients with AD is not due to a disturbance with a specific language process, but may instead be related to other cognitive impairments, in attention, executive function, or praxis.

PROJECT 3: EVALUATING NEUROPSYCHOLOGICAL MODELS OF LANGUAGE RECOVERY WITH FMRI

This past year attention has been focused on examining brain regions that are activated in a short-term memory task using words and word-like strings. The goal is to determine how patients with acquired alexias process orthographic strings, and how such processing changes following successful cognitive treatment of reading.

In a study with normal control subjects, we examined the response to visually presented words and word-like strings during a one-back matching task. The stimuli were (1) common words (e.g. boat) (2) pseudowords (PW), which are pronounceable but not real words (e.g. blink), (3) consonant letter strings (LS, e.g. lxwp), and (4) strings made up of letter like symbols that are unfamiliar to the subjects

(false fonts, e.g. **g**, **u**). We contrasted these tasks with one-back matching of single symbols. We found that all subjects were strongly lateralized to the left occipital/fusiform gyrus in the real words task. No significant differences were found between the word and the PW conditions. The LS condition, on the other hand, produced activations that were somewhat more posterior, bilateral, and parietal than the words and pseudowords tasks, while the FF condition resulted in either right sided or bilateral foci of activations in the occipital and fusiform areas.

The same task was administered to two patients with pure alexia. In this type of reading disorder, written words are no longer readily identified as wholes, and must be deciphered in a serial letter by letter fashion. One explanation for the letter by letter reading seen in pure alexia is that it represents a compensatory strategy implemented by the right hemisphere. Another notion is that it is the result of an inability to access whole word orthographic forms, and reflects reading words as one would "read" an unfamiliar string of letters. The results from the fMRI studies provide partial support for the latter interpretation: When viewing words, the patients with pure alexia show patterns of brain activation that are similar to those seen when normal subjects view consonant letter strings. There is increased left-sided parietal activation, consistent with what is seen when normals view letter strings. No increase in right-sided activation is seen when pure alexia patients view words.

DR. GUOYING LIU, Ph.D.

Dr. Liu's research focuses on both the development and application of functional Magnetic Resonance Imaging (fMRI) of human brain. The general research interests are in a) improved MR methods for functional brain mapping, including the measurement of relative cerebral blood volume (rCBV) and cerebral blood flow (CBF) in health and in disease; and b) the development and the uses of diffusion/perfusion MR methods for the early diagnosis of ischemic stroke and the measurement of the biological activity of experimental stroke therapies.

PROJECT 1: DEVELOPMENT OF MOTION-ARTIFACT INSENSITIVE 3D FUNCTIONAL MRI TECHNIQUES

The potential clinical applications of fMRI techniques are relatively unexplored. The successful application of fMRI techniques in the clinical (as opposed to the research) environment requires the implementation of rapid pulse sequences insensitive to motion-induced artifacts and capable of imaging large volumes of the brain. This project focuses on the development of the 3D volume scanning fMRI technique employing fast imaging and motion reduction techniques, with high efficiency and low image distortion for whole brain 3D functional mapping. The project consists of three parts: A) Development of fast 3D data acquisition techniques in conjunction with the use of echo-shifting to enhance T2* sensitivity, and segmented k-space trajectory to increase the speed. It includes also the development of 3D navigator echo techniques that directly and accurately measure motion induced phase changes, thus allowing motion corrections for rotations and translations in all three directions. B) Evaluation of these techniques for the study of cerebrocortical activity in healthy volunteers. The study uses BOLD fMRI to map patterns of brain activity based on changes in cerebral hemodynamics and perfusion MRI with bolus-tracking to map changes in rCBV. A visual recognition task will be used to assess the sensitivity of these techniques in the detection of areas related to memory. C) Application of these techniques in order to localize brain activity associated with memory deficits in patients with Alzheimer's disease (AD).

It is important to note that studies of functional activation and organization in Alzheimer's disease requires the implementation of rapid pulse sequences insensitive to motion-induced artifacts and capable of imaging large volumes of the brain, as many areas of the brain are included in the studies and as AD patients are much less tolerant and cooperative. It is anticipated that this BOLD fMRI technique should be useful in the clinical investigation of Alzheimer's disease, and will also benefit clinical applications of fMRI to various other categories of neuropsychological and pathological processes in patients.

PROJECT 2: DEVELOPMENT OF SINGLE-SHOT DIFFUSION MRI FOR ACCURATE DIFFUSION MAPPING OF HUMAN BRAIN AND ITS APPLICATION TO ACUTE HUMAN STROKE

Diffusion MRI is a useful method for visualizing early cerebral ischemia and is proving to be an important tool for monitoring noninvasively the progress and treatment of the disease in animal model. Despite a number of successful human applications of diffusion MRI in stroke patients which demonstrate that ischemic lesion can be detected earlier than conventional MRI, diffusion MRI in clinical environments is hampered by its sensitivity to micrometer displacements, and thus, the resulted

motion artifacts. Diffusion-weighted Echo Planner Imaging (EPI) has shown great promise, by demonstrating lesion progression in the penumbra region. However, the expensive instrumental upgrade required for EPI technique and the severe chemical shift and susceptibility artifacts inherent to this technique limit large clinical trials.

This project combines two approaches to overcome the problems of patient motion: 1) single-shot MRI pulse sequence designed for the measurement of diffusion, and 2) advanced navigator motion correction. The basis of the technique is the use of gradient and spin echoes (GRASE) with a modified k-space trajectory and CPMG phase cycle. This single-shot diffusion MR imaging technique with reduced motion sensitivity allows accurate diffusion mapping of human brain with potential application to acute stroke, on an unmodified conventional clinical instrument without any special hardware. In combination with bolus-tracking perfusion experiments using the technique developed in Project 1, rCBV maps and bolus arrival maps are acquired providing useful and complimentary clinical information to that obtained with diffusion imaging in the management of stroke patients.

PROJECT 3: FUNCTIONAL MRI OF PHONOLOGICAL ORGANIZATION IN AUDITORY CORTEX

It has been well established that specific anatomical areas of the superior temporal and inferior parietal cortex, including primary auditory cortex, adjacent auditory association cortex, such as Wernicke's area, and the planum temporale, play an important role in processing speech. The exact relationship between phonological processing of verbal information and the auditory structure has not yet been determined. Our long-term research plan is to seek evidence concerning the location and organization of the cortical regions that transform the acoustic representations of speech into phonological representations. The theoretical premise of our studies is the hypothesis that these transformations involve a spatial remapping of tonotopic representations into phonological representations within auditory cortices.

As the first step towards our overall objective, this study will address three basic aspects of auditory processing in humans, using fMRI: 1) Initial experiments will use simple auditory stimuli (pure-tones) that vary along the frequency axis to define the tonotopic organization of human auditory cortex. In particular, we will seek evidence of additional tonotopic representations outside the primary auditory cortex, and will evaluate the spatial location and boundaries of these multiple tonotopic areas. 2) In contrast to pure tones, activation associated with more complex stimuli, such as band-passed noise bursts and frequency modulated sweeps with differing center frequencies, will be compared as a function of stimulus complexity, thus allowing a systematic investigation of how these cortical areas participate in the hierarchical processing of auditory signals. 3) Finally, using phonemes and scrambled phonemes that are spectrally identical, we will seek to delineate the acoustic features of speech processing and phonological aspects of auditory representations.

The empirical bases for this project are our preliminary fMRI studies of hierarchical processing within human auditory cortex. Our previous human studies demonstrated frequency-specific activation in the primary auditory cortex. Furthermore, we have demonstrated that secondary auditory cortical fields exhibit stronger activation in response to stimuli having phonetic properties of speech in contrast to spectrally identical stimuli lacking the temporal structure of speech sounds.

PROJECT 4: FUNCTIONAL MRI STUDIES OF NEUROANATOMY OF TINNITUS

Tinnitus refers to auditory sensations that cannot be attributed to any external source. It is a severe psychological and clinical problem for which there is, currently, no realistic animal model or objective measurement tool. Although a recent neuroimaging study of tinnitus using PET has demonstrated evidence for limbic system links and neural plasticity, little is known about the brain areas involved and there are few indications for a particular therapy. Our recent work on normal subjects using a 3D fMRI technique demonstrated activation of the superior temporal gyrus in the perception and processing of various auditory stimuli, including pure tones, non-speech noise, and speech sounds. Functional MRI techniques could therefore play an important role in the management of patients experiencing tinnitus.

In collaboration with Dr. Kenneth Grundfast, professor and acting chairman of the Department of Otolaryngology at Georgetown, the research project intends to evaluate these techniques for the study of cerebrocortical activity in patients with auditory cortex pathologies, in particular tinnitus. A special emphasis will be on the effectiveness for the reduction of motion artifacts and image distortion. Using fMRI, we will pinpoint brain regions associated with the existence of tinnitus. To accomplish this goal, the intensity of tinnitus will be varied, and the corresponding change in brain activity will be measured. Previous studies show that broadband white noise and masking can partially alleviate the ringing sound perceived as tinnitus. The results of this study would aid in understanding the mechanism of tinnitus and more importantly may provide a physiologic correlate to measure changes in tinnitus. This marker could then be used to quantify patient response to medications, biofeedback, habituation therapy and other treatments for tinnitus.

INTRODUCTION

Our use of language depends upon a *mental lexicon* of memorized words, and a *mental grammar* of rules that combine lexical forms into larger words, phrases, and sentences. Together, these two capacities provide us with the ability to produce and to comprehend an infinite number of sentences. However, fundamental questions about the brain bases of the two capacities remain unanswered. In particular, two very different theoretical frameworks have been competing for the explanation of their basic functional neuroanatomy. Whereas “dual-system” theories link the lexicon to temporal cortex, and grammar to frontal cortex, “single-system” theories link both capacities to a single network with broad anatomic distribution. Dual-system theories predict dissociations between lexicon and grammar, whereas single-system theories do not. However, testing for lexicon/grammar dissociations has been problematic because tasks probing for lexicon and for grammar usually differ in ways other than their use of the two capacities. We have therefore developed an innovative approach to test the competing views. We have been investigating the brain bases of irregular and regular word transformations, in which lexicon and grammar can be contrasted, while other factors are held constant. Irregular transformations are largely arbitrary (e.g., *cling-clung*, *bring-brought*), and therefore must be memorized in the lexicon, whereas regular transformations (e.g., *look-looked*) can be described by rules of grammar (e.g., an *-ed*-suffixation rule). A dual system view predicts double dissociations between regulars and irregulars, with irregulars (lexicon) linked to temporal cortex, and regulars (grammar) to frontal cortex.

Dual-system theories usually assume components dedicated (“domain-specific”) to language, whereas single-system theories assume general-purpose (“domain-general”) circuitry. However, the dual-system/single-system issue is logically independent from the domain-specific/domain-general issue. We have proposed a third alternative — that *dual* but *domain-general* systems subserve lexicon and grammar. Specifically, we posit that the lexicon/grammar distinction is tied to a fundamental distinction between two well-studied memory systems in the brain: The memorization and use of words depends upon a temporal-lobe “declarative memory” system previously implicated in the learning and use of facts, whereas the learning and use of grammatical rules depends upon a frontal/basal-ganglia “procedural memory” system previously implicated in the learning and use of motor and cognitive skills.

Significance: The proposed research should distinguish among three major theoretical perspectives. If the results confirm the preliminary findings by supporting the novel view that two domain-general brain systems play a role in lexicon and grammar, then data from numerous investigations of the neural, computational, and developmental underpinnings of the two systems in humans and animals may contribute to our understanding of language, and studies of the two language capacities may lead to a better understanding of the two brain systems.

PROJECT 1: NEUROLOGICAL STUDIES OF LANGUAGE AND MEMORY: ALZHEIMER'S, PARKINSON'S, HUNTINGTON'S DISEASE, AND STROKE (APHASIA)

Double dissociations between irregulars and regulars in our studies of patients with impairments of either declarative or procedural memory support this theory, and suggest that the basal ganglia's well-studied role in motor activity extends to grammatical rule use (Love, Hickok, Swinney, & Ullman, 1998; Ullman, in press; Ullman et al., 1997; Ullman et al., in press). Specifically, we asked brain-damaged patients to produce the past tense of regular verbs like "walk" and made-up verbs like "wug" (both of which should require a rule), and of irregular verbs like "think" (which should require memory lookup). We found that patients with damage to brain structures underlying declarative memory (patients with a stroke in left temporal cortex as well as patients with Alzheimer's disease, who have a degenerative disease primarily afflicting the temporal lobes) were worse at producing or reading past tense forms for irregular verbs than for regular or novel verbs. In contrast, patients with damage to brain structures underlying the procedural system (patients with a stroke in left frontal cortex as well as patients with Parkinson's disease, who have a degenerative disease of the basal ganglia) showed the opposite pattern: They were worse producing forms like "walked" and "wugged" than forms like "thought." Unlike patients with Parkinson's disease, whose basal ganglia damage resulted in suppressed movements (trouble starting and carrying out movements) and suppressed rule use ("Yesterday I walk into town"), the different type of basal ganglia damage in patients with Huntington's disease which leads to unsuppressible movements (irrepressible dance-like movements called chorea) also led unsuppressible rule use ("Yesterday I walkededed into town, and dugged a hole").

The demonstration of an analogous dissociation in another language would strengthen the dual-system hypothesis. Italian is a richly inflected language in which a regular/irregular distinction may be found in present tense and past participle inflections. Six Italian patients with probable Alzheimer's disease were asked to produce regular and irregular present tenses ("Mi piace *bere* il vino. Allora ogni giorno _____ il vino") and past participles ("A Giovanni piace *chiudere* la porta. Allora ieri Giovanni ha _____ la porta"). All 6 patients had greater difficulty producing irregular than regular present tenses and past participles (Cappa & Ullman, 1998). These results from English and Italian inflectional morphology link the use of irregulars (lexical memory) but not regulars (grammatical rules) to the use of facts, and are consistent with a role for temporal lobe circuits in word and fact use.

We propose to confirm and extend these findings. Patients with either temporal-lobe or frontal/basal-ganglia damage will be asked to produce and judge irregular and regular past-tenses (*sleep-slept/slip-slipped*), plurals (*mouse-mice/bee-bees*), and derivational forms (*solemn-solemnity/eager-eagerness*), and will also be given tasks probing fact and skill use.

Hypothesis 1. Temporal-lobe circuits underlie word use, frontal/basal-ganglia circuits underlie grammatical rule use: Irregular/regular double dissociations will be tested between (a) subjects with left temporal-lobe damage and probable sparing of frontal/basal-ganglia structures (in posterior aphasia and Alzheimer's disease); and (b) subjects with left frontal/basal-ganglia damage and probable sparing of temporal regions (in anterior aphasia and Parkinson's disease).

Hypothesis 2. The circuits subserving word use also subserve fact use; the circuits subserving rule use also subserve skill use: Associations will be tested between deficits in the use of facts and of

irregulars in posterior aphasia and Alzheimer's disease, and between deficits in the use of skills and of regulars in anterior aphasia and Parkinson's disease.

Hypothesis 3: Basal ganglia pathways play similar roles in motor activity and grammatical rule use: In subjects with Parkinson's disease or Huntington's disease, associations will be tested between (a) hypokinesia and suppressed suffixation; and (b) hyperkinesia and unsuppressed suffixation.

PROJECT 2: EVENT RELATED POTENTIAL (ERP) STUDIES OF LANGUAGE AND MEMORY

We have been investigating the brain bases of lexicon and grammar using the electrophysiological technique of measuring the Event Related Potentials (ERPs) associated with the presentation of stimuli. Previous evidence had suggested that lexical or semantic violations yield central/posterior negativities ("N400") (Hagoort & Kutas, 1995), whereas grammatical violations can yield left anterior negativities ("LAN") (Neville, Nicol, Barss, Forster, & Garrett, 1991). Unfortunately, the tasks tapping these lexical and grammatical capacities have not been well-matched in factors other than the two capacities. Therefore we have been investigating the electrophysiological basis of lexicon and grammar by probing the use of irregular and regular forms.

Right-handed males viewed sentences that appeared, one word at a time, on a monitor, while ERPs were being recorded. In Experiment 1, all sentences were framed in the past tense. Subjects were shown 64 regular verbs matched on frequency, orthography, and phonology to 64 irregular verbs. Half of each were presented in the correct past tense form (Yesterday I looked at Sue), and half in the stem form (Yesterday I walk after lunch), the latter being a morphological violation (Newman, Neville, & Ullman, 1998). In Experiment 2, these sentences were intermingled with sentences which contained either errors of meaning (e.g., I drank my coffee with *dog*) or of sentence-level grammar ("syntax") (e.g., I drank my *with* coffee sugar) (Newman, Izvorski, Davis, Neville, & Ullman, 1999). We found that violations of regular verb inflection and of syntax yielded a left anterior negativity (LAN), while violations of irregulars and of meaning elicited enhanced negativities with a right-lateralized, more central distribution (an N400) (Newman et al., 1999; Newman et al., 1998) (see Figure 1).

These experimental results indicate that lexicon and grammar are associated with distinct electrophysiological signals and subserved by distinct neural processes. They link regular morphology to syntax, and irregular morphology to lexical-semantic processing. Moreover, because the N400 pattern is associated with left temporal-lobe sites (Nobre, Allison, & McCarthy, 1994; Papanicolaou, Simos, & Basile, 1998), the findings also link irregular morphology to left temporal lobe structures.

In the next two years we will further investigate the electrophysiological basis of grammar and lexicon. First, we will attempt to replicate the preliminary results described above, using our newly-built 96-channel ERP system at GICCS. (The studies above were run on a 32-channel system). This allows for higher electrode densities, and therefore provides the means for better characterizing the spatial distribution of the ERP components. Second, we will test the same subjects on several ERP syntactic tests which we have already constructed, as a means of further characterizing the neural basis of grammar. We hypothesize that the LAN component emerges from violations of concatenation. Therefore we predict that only grammatical violations of concatenation, and not other types of grammatical violations (e.g., of movement), will lead to a LAN. Third, we will test the Alzheimer's and Parkinson's patients who have already been tested behaviorally in Project 1 on the same ERP task.

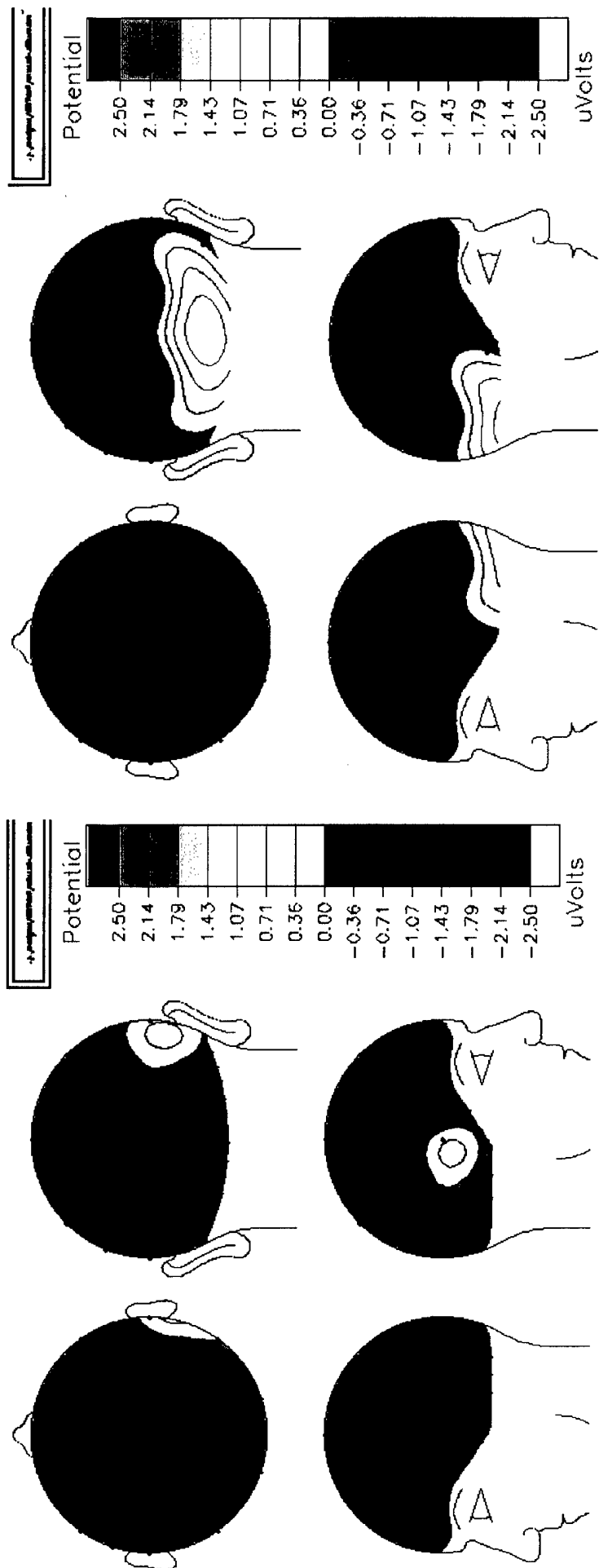


Figure 1: Voltage maps associated with the presentation of incorrect vs. correct regular (left) and irregular (right) past tense forms.

This will provide a means of investigating the neural basis of language, its impairments, and its preservation, in each of the patient groups tested in Project 1. We hypothesize that patients with temporal lobe damage and lexical-semantic impairments (those with Alzheimer's disease or posterior aphasia) will lack an N400 but not a LAN, whereas patients with frontal/basal-ganglia damage and grammatical impairments (those with Parkinson's disease or anterior aphasia) will lack a LAN but not an N400.

PROJECT 3: FUNCTIONAL MAGNETIC RESONANCE IMAGING (FMRI) OF LANGUAGE AND MEMORY

We have also been investigating the brain bases of lexicon and grammar with functional brain imaging. Five healthy right-handed men were shown the stems of irregular verbs (*sleep*) and non-rhyming regular verbs (*slip*) on a screen, and were asked to silently produce their past tense forms. Twenty seconds of regulars (10 verbs) were followed by 20 seconds of fixation, 20 seconds of irregulars (10 verbs), and 20 seconds of fixation. This sequence was repeated for 80 irregular and 80 regular verbs. Functional scans were obtained using an Asymmetric Spin Echo pulse sequence with a TR of 2 seconds. We examined 12 axial oblique slices spanning the frontal, temporal, and occipital lobes, and the cerebellum. All functional data were motion-corrected using a modification of the AIR algorithm. Functional data from each subject were then transformed into Talairach space. Signed Kolmogorov-Smirnov statistics were calculated for three comparisons: Regular vs. Fixation, Irregular vs. Fixation, and Irregular vs. Regular. In addition to examining the data from each individual subject, the Talairach transformations allowed us to combine the data from all five subjects.

The five subjects showed similar patterns of activation. We found overlapping as well as distinct patterns of brain activation for the irregular and regular conditions. All activation changes were confirmed with time-course analyses (Figure 1). Compared to fixation, both the irregular and regular conditions yielded activation increases in inferior frontal regions (including Broca's area), and in the basal ganglia (in the caudate nucleus). This suggests that these regions may subserve a function common to both past tense types, such as the computation of the grammatical tense feature (Jaeger et al., 1996).

Left temporal and temporo-parietal regions were associated with an activation decrease for irregulars, but not for regulars. In contrast, a left prefrontal region was associated with an activation increase for irregulars, but a decrease for regulars. Interestingly, the activation increase in left prefrontal cortex, and decrease in superior temporal cortex associated with irregulars, but not regulars, was also observed during a verbal fluency task (generating words in a category) by Wise et al. (1991). While the specific causes of these activation changes remain to be investigated, the double dissociations suggest that irregulars and regulars have distinct neural underpinnings linked to temporal and frontal regions. It is also of interest that the patterns of activation reported for a recent PET study of German regular and irregular past tense processing (Indefrey et al., 1997) are similar to those found in this study, and therefore may also in part be explained by activation decreases.

We have also developed a task in which regular and irregular verbs are presented in a randomized rather than a blocked sequence. We have acquired images after each verb, thus treating

each verb as a distinct trial. Such "single-trial fMRI" (also termed "event-related fMRI") is a promising new technique which may allow us to observe the time course for each linguistic event.

The technique avoids the problems inherent in presenting blocks of stimuli of the same condition. In the next two years we will replicate our ERP language studies described above with event-related fMRI. Because of the very high spatial resolution of fMRI, this is potentially an extremely important endeavor, because it may begin to identify the neural loci of ERP language components that have been very well studied, but whose neuroanatomical substrate is unknown. Finally, just as with ERP, we will be testing anterior and posterior aphasics, and patients with Alzheimer's or Parkinson's disease, to further characterize the neural basis of their language in these neurodegenerative diseases.

PROJECT 4: MAGNETOENCEPHALOGRAPHIC (MEG) STUDIES OF LANGUAGE AND MEMORY

Magnetoencephalography (MEG) provides a method to investigate the real-time spatio-temporal dynamics associated with the production of regular and irregular past tense forms. We are collaborating with scientists at MIT, where there is a whole-head 64-channel magnetometer. Recently we recorded from the magnetometer while subjects produced past tenses of regular and irregular verbs (Rhee, Pinker, & Ullman, 1999). Satisfactory solutions to the inverse problem of dipole fitting for data averaged over all subjects were found at a number of 10 millisecond time-slices following stimulus presentation. No right-hemisphere dipoles were found. Dipoles in both the regular and irregular verb conditions were localized to a single left temporal/parietal region (250 to 310 milliseconds). Dipoles in left frontal regions were found only for regular verbs, and only for time-slices immediately following the left temporal/parietal dipoles (310-330 milliseconds). The results are consistent with a dual-system model in which temporal/parietal-based memory is searched for an irregular form, whose successful retrieval blocks the application of a frontal-based suffixation rule (Ullman et al., 1997).

Over the next two years we will be running a replication of our ERP and fMRI studies of both unimpaired and cognitive impaired subjects with MEG. Because MEG allows for far better source localization than does ERP, while *also* providing real-time temporal resolution, this may allow us to pinpoint not only the anatomical source of the well studied ERP language components, but also their real-time pattern in the brain, providing a "movie" of the computation of linguistic forms.

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ANIMAL COGNITIVE NEUROSCIENCE: Dr. Kanwal investigates the processing of communication sounds and modeling of a neural network for multidimensional analysis of speech sounds and pattern recognition. Dr. Rauschecker examines the functional organization and plasticity of the cerebral cortex with an emphasis on the auditory cortex and multi-modal processing using single-neuron microelectrode recording, optical recording, and functional MRI. Dr. Wu studies large-scale neuronal events and distributed processes in the CNS.

JAGMEET KANWAL, Ph.D.

The major goal of Dr. Kanwal's research is to elucidate normal auditory processes involved in the coding/decoding and perception of communication sounds. The dogma challenged by his research is that animal studies can teach us little, if anything, about two of the most highly specialized functions of the human CNS, namely, perception of speech and music - two related facets of human cognition that make us unique from all other animals. It appears, however, that to really understand the neural mechanisms and neurophysiological principles underlying speech-sound perception one must study its minimal analogs present in an animal CNS. This has led to significant progress in the last decade, in our understanding of early auditory processing that underlies speech production as well as perception in humans. Similarly, basic phenomena such as perception of the "missing fundamental" that can be equated to certain aspects of music perception have been recently demonstrated at the psychophysical and neurobiological levels in bats and primates, respectively.

Dr. Kanwal's research uses multiple approaches to investigate auditory processing within higher levels of the CNS in auditorily specialized animals, such as bats and humans. These approaches include single cell electrophysiology, evoked potentials and ERPs and functional MRI as well as behavioral techniques. A quantitative methodology incorporating advanced statistical analyses is stressed. One electrophysiology set-up is up and running and a second one is potentially functional. This is presently being used for conducting acoustical and behavioral analysis of communication sounds. Dr. Kanwal together with his colleagues enabled GICCS to set up a core histology/imaging facility that will open up new avenues of research. Results obtained from several ongoing/completed research projects, supported in part by the Institute are categorized under three themes and briefly described below:

THEME 1: AUDITORY COMMUNICATION IN BATS: ACOUSTICS, BEHAVIOR AND NEUROPHYSIOLOGY.

Introduction

Mustached bats depend largely on their auditory systems to survive in their ecological niche just as humans are uniquely equipped for survival by their ability to communicate acoustically. The significance of the single development of "speech" in humans is especially clear when one considers human evolutionary history. For several reasons, the auditory system of bats is an excellent model to study neural mechanisms that may underlie processing of communication sounds in mammals, including speech sounds in humans. For example, recent studies suggest that the bat's cortex shows hemispheric asymmetry for processing communication sounds. In addition, the presence of combination-sensitive neurons and multidimensional representational schemes for processing complex syllables in the auditory cortex may be applicable to speech sound processing as well. Not surprisingly, the field of communication sound processing has re-emerged as an actively growing field as it attempts

to understand the neural basis of speech perception and its commonalities with the audio-vocal system of mammals at the single neuron level. Bats are useful models also because they employ a complex repertoire of sounds for acoustic communication rivaling that of the most vocal primate species. Even though the auditory system of microchiropteran bats is the best studied among mammals, previous neurophysiological studies on audition in bats have mostly focused on their echolocation behavior.

Continuing Projects:

PROJECT 1A: ACOUSTIC COMMUNICATION BEHAVIOR IN MUSTACHED BATS

Mustached bats, *Pteronotus parnellii*, have been used extensively for studying neural and behavioral aspects of echolocation and recently for research on neural mechanisms for processing communication calls. Even though the acoustic structure of their communication sounds has been described in detail, information on the association of these sounds with different social behaviors is lacking. We describe here "fixed action patterns" or visually explicit stereotypic behaviors some of which accompany social and vocal interactions. Using synchronized audio-video recordings, we obtained evidence for the association of specific syllables, e.g., fixed Sinusoidal Frequency Modulation and rectangular Broadband Noise Burst syllables, with aggression. Furthermore, we manipulated the social and auditory environment of captive *Pteronotus* to establish that stereotypic behaviors vary with group dynamics and that aggression is frequently associated with and can be influenced by specific calls. A paper is nearly ready for submission to the Journal of Comparative Psychology.

Additional work in this area has led to the development of a behavioral assay that will allow us to examine the ability of bats to discriminate different sounds as well as the role of different brain areas for social communication. We are particularly interested in role of the frontal cortex in different social communication behaviors as this area appears to be crucial for transforming sensory information into motor behaviors.

PROJECT 1B: CALL PROCESSING IN THE FRONTAL CORTEX OF MUSTACHED BATS

Response properties of neurons in an auditory field in the frontal cortex of the mustached bat, *Pteronotus parnellii*, have not been studied before. We recorded neural responses to constant frequency (CF) stimuli from the frontal auditory field in awake animals. The majority ($\approx 75\%$) of neurons in this area responded well and often exhibited low thresholds to CF stimuli. Most CF-responsive neurons exhibited sharp tuning with $Q_{30\text{ dB}}$ values of over 180. Neurons at thirteen recording sites exhibited combination-sensitivity in that their responses were facilitated by presenting combinations of either CF_1/CF_2 and/or CF_1/CF_3 components of the mustached bat's echolocation signal. Unlike the typical on-responses to a 30 ms tone, observed in the mustached bat's auditory cortex and at subcortical levels, many frontal auditory neurons exhibited loosely time locked firing patterns that lasted for over 100 ms.

Auditory inputs are transmitted to the frontal cortex via two thalamocortical pathways. One is a lemniscal projection from the brainstem via the medial geniculate body (MGB) and the auditory cortex and the other is a more direct extralemniscal projection from the supragenulate nucleus in the thalamus. Processed auditory information can thus reach the frontal cortex via the latter pathway in as

few as four synapses. Despite these two ascending auditory pathways to the frontal cortex and its reciprocal connections to the auditory cortex, we know very little about the nature of auditory processing within this brain region in any mammalian species.

Data on auditory responses from neurons within the frontal cortex of the mustached bat are particularly useful because this species has become an excellent model for studies of echolocation and auditory processing in general. Only recently have attempts been made to systematically study auditory responses within this part of the cortex in the mustached bat, *Pteronotus parnellii*, even though this area/field was identified in the mustached bat's brain several years ago. For echolocation, the mustached bat emits sounds consisting of four harmonics (H_1 - H_4), each with a constant frequency component (CF_1 - CF_4) and a downward-sweeping frequency modulated component (FM_1 - FM_4). The CF components are harmonics of an approximately 30 kHz fundamental.

We have described the basic stimulus-response properties including excitatory and facilitatory tuning and the temporal patterns of auditory responses to tones, multi-tone complexes and tone combinations that correspond to the CF component within species-specific echolocation sounds. Studies on the auditory processing of communication sounds emitted by this species have also yielded new insights to neural specializations in the auditory cortex. We are beginning to study their processing in the frontal cortex as well, but these data go beyond the focus of this report.

Figure 1 shows representative peri-stimulus-time-histograms (PSTH's) of tone-responsive neurons in the frontal cortex. A: PSTH's were computed from single neuron responses to 30 ms tone-bursts generated at the neurons' best frequency (binwidth = 5 ms; SPL = 35 dB above minimum threshold, stimulus repetition rate = 2/s). Top: PSTH of a response to 61.6 kHz at 35 dB SPL, Middle: PSTH of a response to 61.2 kHz at 45 dB SPL, and Bottom: PSTH shows a double-peaked response to 61.6 kHz at 35 dB SPL. All stimuli were within 300 Hz the resting CF2 of the bat. B: PSTH's for a combination-sensitive (CF/CF) neuron. Top to bottom: spontaneous activity, response to CF_1 (25.77 kHz, 67 dB SPL), response to CF_2 (61.81 kHz, 7 dB SPL), and facilitated CF_1/CF_2 response (binwidth = 5 ms, stimulus duration = 30 ms, stimulus repetition rate = 3/s, stimulus repetitions = 1000). Stimulus onset and duration are indicated by horizontal lines; acoustic delay between speaker and the bat's ear is considered. Note the presence of a damped oscillation in the CF_1/CF_2 response.

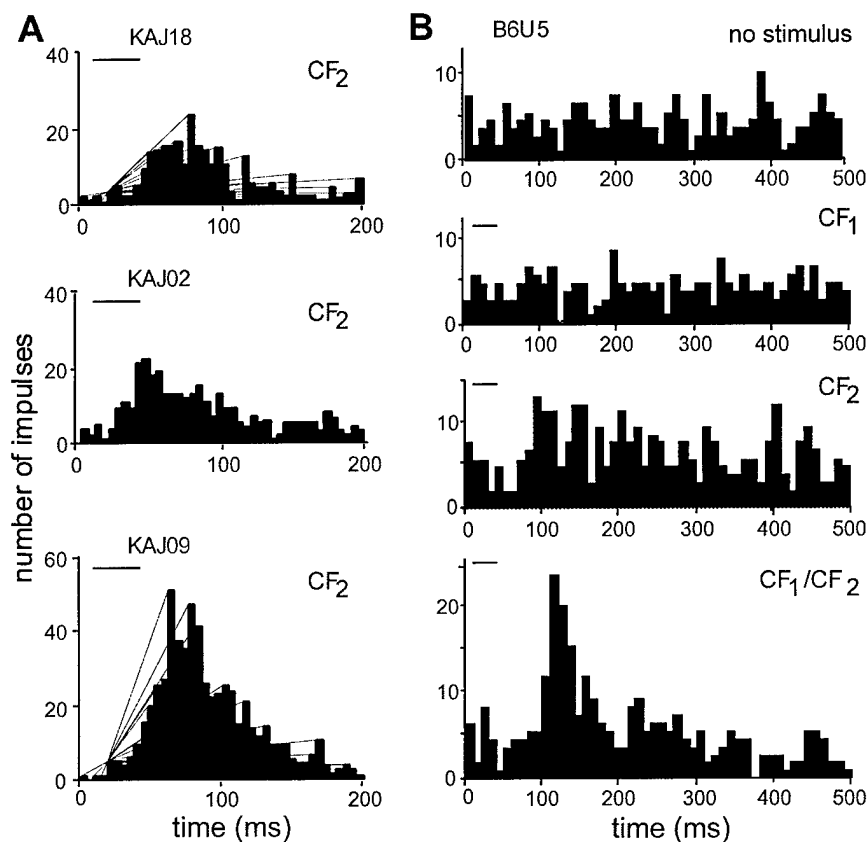


FIGURE 1

THEME 2: MUSIC PERCEPTION IN HUMANS: ACOUSTICS, NEUROPHYSIOLOGY AND IMAGING.

Introduction

Early psychophysical studies on music perception have yielded interesting data. This independent though related line of research is being pursued in parallel with other studies. Music is the combination of complex and pleasant vocal/instrumental sounds or tones consisting of rhythm, melody and harmony. Music can facilitate all biological drives and motivation and generate states of relaxation and ecstasy which play an important role in creating a sense of well being. The underlying neural basis of these effects, however, is largely unexplored.

PROJECT 2: IMAGING HUMAN CORTICAL AREAS INVOLVED IN PERCEPTION OF SPEECH, ENVIRONMENTAL AND MUSICAL SOUNDS.

Research in this area has been slow because of limited support available from DOD funds in the last funding cycle. However, we have recently made progress in the analysis of data on musical

imagery, especially in musically trained subjects. These data clearly show specific areas that are activated by the imagery of music. Surprisingly, areas in the auditory cortex, visual cortex and the cingulate region show the greatest activation. Conclusive results cannot be described here until all of the analysis is completed.

Audio Sequencing And Presentation (ASAP)

Load an experiment Save this experiment

No. of repetitions: Initial delay (ms):

☒ Free-run ☐ Do not generate triggers
☐ Trigger experiment ☒ Generate start trigger
☐ Trigger each block ☐ Generate block triggers

Latency (ms): Latency (ms):

	File Name	Block Duration (s)	Sound Rate (Hz)	Silence Interval (ms)
1	art.txt	10	1	50
2	env.txt	10	1	50
3	nat.txt	10	1	50
4				
5				
6				

art.txt
 env.txt
 guitmri.txt
 guitmri1.txt
 guitmri2.txt
 guitmri3.txt
 mahler.txt
 mahler1.txt
 mahler2.txt
nat.txt
 silence.txt

Prepare [Playback Controls]

Laboratory for Auditory Communication and Cognition

THEME 3: TECHNOLOGICAL INNOVATIONS: SOFTWARE AND HARDWARE ENGINEERING

“Human Auditory Stimulation” software for fMRI studies for Windows 95™

This software designated as “ASAP” has been completed and a paper submitted for publication in the J. for Neurosci. Methods.

A computer program, named ASAP (for "Audio Sequencing and Presentation"), has been developed for IBM PC-compatible systems operating under Microsoft Windows. ASAP's purpose is the sequencing and presentation of complex sounds for auditory neuroimaging studies. The development of ASAP fills a void in the limited software that is presently available for this purpose. This program achieved several objectives in order to best meet the needs of researchers. The main objective was precise control of the time of delivery of each sound. This is critical for most studies of auditory processing. ASAP frees the researcher from manually attending in a timely fashion to sound playback devices. The ability to trigger and to be triggered by data acquisition devices is important for both the automation of any study and for synchronizing stimulus presentation with the data acquisition devices. An example of ASAP triggering a Siemens MAGNETOM Vision System (1996) is provided in the next section. Another objective was the capability of presenting either the same stimulus or different stimuli to each ear, i.e., monotic or diotic stimuli. Pre-filtered sounds may also be assembled to generate dichotic stimuli (part of the stimulus exciting one ear and the rest of the stimulus the second ear). These are useful for studies of hemispheric asymmetry. Finally, the capability of presenting visual stimuli is sometimes required in auditory studies; e.g., the presentation of instructions to the subject. ASAP can display pictures and text at any location on the video display and at any time during auditory stimulation.

FUTURE DIRECTIONS

Data obtained from the above described studies on communication sound processing will allow this laboratory to explore novel mechanisms and formulate new principles of auditory processing, especially at the cortical level. The long-term goal is to elucidate both similarities and differences in communicative auditory processing at the cellular and systems levels between animals and humans.

JOSEF P. RAUSCHECKER, Ph.D., D.Sc.

PROJECT 1: CORTICAL MECHANISMS OF AUDITORY PROCESSING IN NONHUMAN PRIMATES

Project 1A: Processing of Complex Sounds in Nonprimary Auditory Cortex

Dr. Rauschecker's group continues to explore the functional organization of nonprimary auditory cortex in macaque monkeys. Further analysis of single unit responses to complex auditory stimuli in different cortical fields has provided the following highlight of this year's research: We were systematically comparing the response selectivity of single neurons in the anterior and caudal belt regions of rhesus monkeys to (a) species-specific vocalizations and (b) spatially localized sounds, in order to see if these regions could form the origin for two separate processing streams. A significant trend was found for neurons in AL to be more highly selective in their response to monkey calls than neurons in CL ($p < 0.05$, Kruskal-Wallis test). By contrast, neurons in the caudal region were overall much more specific for the spatial location of a broad-band sound presented in free-field, confirming earlier reports of spatially tuned neurons in area Tpt of caudal superior temporal cortex ($p < 0.001$, Kruskal-Wallis test). Tuning to the rate of frequency-modulated (FM) sweeps was also different in areas AL and CL: Neurons in AL prefer comparatively slow FM rates, as they are contained in monkey calls, whereas CL neurons prefer fast FM rates which, due to their broader frequency spectrum, are apt for sound localization. These findings are important because they provide strong electrophysiological evidence, for the first time, that the auditory cortical system is organized into two processing streams, one for the processing of auditory patterns, including communication sounds and speech (in humans), and one for the analysis of auditory space.

Project 1B: Neuroanatomical Connectivity of Auditory Cortex

The processing hierarchies within primate auditory cortex were also further investigated with neuroanatomical tracer studies. During the past year, efforts concentrated on the analysis of cortico-cortical connections from the superior temporal gyrus (STG) to the prefrontal cortex. Tracer injections were made into physiologically identified areas of cortex. Quantitative analysis with the NeuroLucida system again shows that the frontal connections from STG are organized into separate dorsal and ventral streams, similar to the visual system, which underlie spatial and pattern processing, respectively (Romanski et al., 1999).

PROJECT 2: FUNCTIONAL MRI OF AUDITORY CORTEX IN HUMANS

Over the last year, we have again expanded our program of studies using fMRI of human auditory cortex on the 1.5T Siemens Vision scanner at Georgetown. We have concentrated on the use of human speech sounds ("phonemes" or "consonant-vowel [CV] combinations"), which lead to highly specific and highly reproducible activation in areas of the superior temporal cortex (Wessinger et al., 1998; Morad et al., 1999). The organization of higher auditory areas involved in speech processing into phonemic maps or phonemic columns is the main focus of our attention at the moment.

There is also evidence that what we see influences what we hear. In the "McGurk effect" a lack in congruence of lip movements and sounds originating from a face leads to the perception of a different speech sound. This demonstrates that visual and auditory portions of the speech signal are integrated into a common percept in the brain. Our fMRI data with this paradigm demonstrate specific activation in the parieto-occipital region during visual-auditory fusion as well as deactivation in superior temporal regions of the so-called fusiform face area.

PROJECT 3: COMPENSATORY PLASTICITY OF THE CEREBRAL CORTEX

Our continuing collaboration with the group of Dr. Mark Hallett at the National Institute of Neurological Disorders and Stroke (NINDS), using positron emission tomography (PET), has produced two published papers during the past year as well as a submitted manuscript. The findings are relevant for an understanding of the organization of higher auditory processing in the human brain and its plasticity. Sound localization of virtual auditory space stimuli activates two centers in the posterior parietal lobe, one of them modality-specific for auditory spatial processing, the other activated by both visual and auditory spatial tasks (Weeks et al., 1999; Bushara et al., 1999). The plasticity of this system had can be demonstrated by the fact that the area of activation in the parietal cortex is significantly enlarged in congenitally blind people and extends far into normally visual areas (18 and 19) in the occipital lobe (Weeks et al., 2000).

The plasticity of auditory cortex has been discussed in several invited review articles written by the PI over the last year, most notably one in *Trends in Neurosciences* (Rauschecker, 1999a) and one in *Science* (Rauschecker, 1999b). The findings discussed in there should be valuable tools for the study of other forms of neural plasticity and recovery of function in patients with central neural injury and brain damage. In particular, understanding of cortical plasticity is necessary for development of treatment of patients with stroke or Alzheimer's disease.

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JIAN-YOUNG WU, Ph.D.

Dr. Wu's laboratory studies neuronal activity in neocortex. There are about one trillion neurons in neocortex. These neurons are simple devices and each can represent only a small portion of the outside world. However, human consciousness is generated by the collective activity of these neurons. Thus, one ultimate goal for system neurobiologists is to understand how complete and complex cognition develops from a simple and partial representation of individual neurons.

My research fits into an intermediate step between neurons and brain-- the role of dynamic neural assemblies which are subconscious functional units with complex internal representations. My laboratory uses optical imaging to analyze the population neural events in neocortex. About sixty years ago, Sir Charles Sherrington, a neuroscientist (and Nobel laureate) had imagined a fascinating way of viewing the brain activity:

"Imagine activity in (the brain) shown by little points of light. Of these some stationary, flash rhythmically, faster or slower. Others are traveling points, streaming in serial trains at various speeds. The rhythmic stationary lights lie at the nodes. The nodes are both goals whither converge, and junctions whence diverge, the lines of traveling lights."

At that time brain imaging was only a dream. After years of continuous effort from a number of laboratories, optical imaging has become a powerful technique. Imaging population activity rather than recording from individual neurons (a metaphor would be viewing a whole TV screen versus analyzing the intensity of individual pixels) now has a unique position in system neuroscience.

Voltage sensitive dye imaging, the major approach of my laboratory, directly reports the electrical activity of the neurons and can have a temporal resolution of 0.5 ms, enough to follow dynamic neural events. Currently we have two major projects in my laboratory. One is aimed at understanding the localized dynamic neural assemblies, and the other is aimed at understanding the initiation site (as a pathological neural assembly) of epileptiform activity. From the information included in this report you can see that we are able to record the activity of small groups of neurons and study their roles in cortical processing and epileptogenesis.

PROJECT 1: DYNAMIC CORTICAL NEURAL ASSEMBLY

The long term goal of this project is to understand the basic building blocks of population activities in neocortex. We are studying a propagating event in cortical slices. These local population events occur during 7 to 10 Hz oscillations. They can also be evoked in cortical tissue when a small number of neurons are activated by electrical stimulation. Throughout the proposal we use the words, "dynamic ensemble" as the name for this phenomenon. Our goal in 1999 was to distinguish it from known population events such as epileptiform activity and evoked population response. Most of the experiments have been reliably repeated and published in abstract forms (Wu et al 1997). Some data is published (Wu et al 1999) or accepted for publications (Tsau et al 1999) or submitted (Wu and Guan 1999, Bolea et al 1999).

Evoking dynamic ensembles

Dynamic ensembles were evoked in cortical slices perfused with normal ACSF. Specific methods of stimulation are needed to properly activate the neuronal network; we know of three which can evoke dynamic ensembles in somatosensory and auditory cortices.

One method is to activate a low density of neurons in a large area which includes multiple cortical layers (Wu et al., 1999). A single pole electrode placed 100 to 300 μm above the tissue surface is used to activate the neurons in a large area including multiple cortical layers. The area of activation and density of activated neurons can be independently adjusted by changing the distance between the electrode and the tissue surface and by using different stimulus intensities. Optical imaging showed that a low level of activation (0.3 to 3 times the local field potential response threshold) distributed in an area about 1- 3 mm in diameter was most likely to evoke dynamic ensembles. The intensity of stimulation is extremely important in evoking dynamic ensembles. A near-threshold stimulus applied to an area may differentially activate different types of neurons, thereby creating a substantial population effect for developing dynamic ensembles. In contrast, conventional stimulation (a thin coaxial electrode placed in the deep layers) usually activated a small area with a high density of active neurons and was not able to evoke dynamic ensembles.

Stimulating the thalamic afferent fibers to the cortex (Metherate and Cruikshank, 1999) is a second method for evoking dynamic ensembles. We used two cortical slice preparations in which thalamocortical afferent fibers are preserved - slices from somatosensory area (Agmon and Connors, 1991) and auditory cortex (Metherate, 1997). A thin coaxial stimulus electrode was used to shock the fiber bundles leading from the thalamus to the cortex. A single shock of moderate intensity is enough to evoke a dynamic ensemble which is sustained for several hundred ms.

Stimulating the corticocortical projections in layer IV is the third way to evoke dynamic ensembles (Hsieh et al., 1998). All three methods of stimulation evoked dynamic ensembles with the same major features; they were all-or-none events, self-sustaining with large trial-to-trial variabilities in local field potential signals. Dynamic ensembles evoked by these three methods were indistinguishable, which may be partly due to the large trial-to-trial variability of the dynamic ensembles.

Distinguishing dynamic ensembles, "passive" response, and epileptiform activity

In LFP recordings dynamic ensembles appeared as low amplitude population activities, usually emerging at 20 to 100 ms post-stimulus and lasting 50 to 400 ms (250 ± 100 ; $n = 620$ ensembles from four preparations), with large trial-to-trial fluctuations in waveform. Dynamic ensembles were found to be all-or-none population events; identical stimuli at near-threshold intensity either generated dynamic ensembles or failed to do so. In contrast, a passive response was composed of neurons directly activated by the electrical shock. Passive responses had a shorter and more certain latency (2 to 5 ms) and a shorter duration (~ 10 ms). The amplitude of a passive response increased with the stimulus intensity until reaching a plateau. In contrast, dynamic ensembles had a low amplitude with large fluctuations; their waveform had no correlation to stimulation intensity. These features -- all-or-none, variable latency, prolonged duration and large fluctuating waveform -- suggest that a dynamic ensemble is not a direct response to the stimulus, but rather it is likely to be an emerging activity of the local neuronal

network. The long duration of dynamic ensembles further suggests that the activity is self-sustained by mutual excitation among neighboring neurons and/or inhibition of inhibitory neurons.

The stimulus intensity is crucial for evoking dynamic ensembles. The optimum stimulation intensity is at or lower than the threshold for a visible passive response in the local field potential threshold. With a proper intensity a large portion of the stimuli evoked dynamic ensembles. Surprisingly, increasing the stimulus intensity substantially reduced the probability of inducing dynamic ensembles. Using single pole electrode and a near-threshold stimulus, neurons distributed over a large area may be activated; within the area, different types of neurons may be activated differentially due to the variety of geometric shapes and distribution of the processes. Such differential activation of a large population may create substantial population effects for dynamic ensembles to develop.

The activity of dynamic ensembles could be clearly distinguished from that of epileptiform events. The latter had a much larger amplitude in the LFP, was higher in propagation velocity (Tsau et al., 1998; Demir et al., 1999), and had less fluctuation in its waveform. When a low dose of bicuculline (final concentration $< 2 \mu\text{M}$) was added to the perfusing media, an optimum stimulus failed to evoke dynamic ensembles ($N=240$, three preparations). At this concentration of bicuculline, epileptiform activity could not be triggered, thus only a passive response could be seen. Epileptiform events occurred when a higher dose of bicuculline was perfused. These results strongly suggest that dynamic ensembles are different from epileptiform events.

Spatiotemporal patterns of dynamic ensembles

Voltage-sensitive dye imaging showed that dynamic ensembles are self-sustaining, which distinguishes them from the passive response to a stimulus. A passive response and a dynamic ensemble were elicited by a single pole electrode (stimulation method 1, see Methods). A passive response started from the stimulation site and propagated quickly in the tissue. When a passive response failed to develop into a dynamic ensemble, the activity decayed within 50 ms. In contrast, dynamic ensembles emerged 20 to 200 ms post-stimulus, usually initiating from the edge of the area activated by the stimulus. Using the 50% contour line of the amplitude of the voltage-sensitive dye signal (provided by NeuroPlex), we estimated that the dynamic ensemble was about 0.5 mm wide in the tangential direction in coronal slices. The activated area usually included all cortical layers; sometimes the duration of the activity appeared to be longer than that measured by LFP signals, because the activity propagated over a large area and the electrode only detected part of the activity. Comparing simultaneous LFP and optical recordings, we found that the waveform of the LFP had much larger fluctuations than that of voltage-sensitive dye signals from the same location. This will be discussed in the next section.

Dynamic ensembles propagated in tangential directions. During propagation the area of activation did not expand. Propagation without expanding further suggests that dynamic ensembles are not sustained by fixed, hardwired connections. The overall horizontal propagation velocity had a large range (4- 17 mm/sec, $n= 24$ trials from 4 preparations). The propagation velocity was unevenly distributed along the path, in some locations very low (0-1 mm/sec) and in others significantly higher (~ 30 mm/sec, data not shown). In contrast to the slow and variable propagation in tangential directions, propagation in the vertical directions appeared to be much faster (50 - 100 mm/sec). This directional selectivity is similar to the propagation of co-activation (the giant depolarizing potentials, GDP, Ben-Ari et al., 1989) in the CA3 areas of developing hippocampus (S. Bolea, L. Guan, J.V. Sanchez-Andres,

and J.-Y. Wu, unpublished observations). Uneven propagation velocity in a tangential direction was also seen during the propagation of epileptiform activity (Chervin et al., 1988; Wadman and Gutnick, 1993) and oscillations in neocortex (Wu et al., 1999), and during the propagation of network co-activation in the developing hippocampus (Bolea et al., unpublished observations).

The density of active neurons in a dynamic ensemble was estimated from the amplitude of voltage-sensitive dye signals. The highest amplitude evoked by a large electrical shock was taken as "maximum activation". We assume that in the area of maximum activation the majority of neurons around the tip of the stimulating electrode were simultaneously (within 1 millisecond) activated, orthodromically or antidromically, by the shock. The amplitude of the optical signal of a dynamic ensemble was about 5 to 7 percent of the amplitude of maximum activation (data not shown). This suggests that the dynamic ensemble is an area with moderate depolarization and a low firing probability of the participating neurons.

Trial-to-trial and location-to-location variations

In LFP recordings the dynamic ensembles had a large trial-to-trial variability. Trial-to-trial variations could be seen on two temporal scales. On a scale of 1-10 ms, the waveform of LFP signals was highly variable in different trials evoked by identical stimuli. The waveform in LFP covered a wide frequency spectrum, with noticeable peaks around 12, 40 and 80 Hz. The same network simultaneously oscillating at different frequencies was also observed in the carbachol-induced oscillation in adult hippocampal slices, where 40 Hz oscillations exist at the same time with theta (~10 Hz) rhythms (Fisahn et al., 1998). On a larger temporal scale of 50 to 100 ms, we found that identical stimuli could evoke a response consisting of multiple bursts of activation. Both duration and intervals of the bursts were variable and sometimes a long activity appeared to be composed of smaller bursts. The repeating frequency of the bursts was 4 to 10 Hz (N=10).

Multi-electrode recordings revealed that at different recording locations the LFP signals could have large location-to-location variations. While some of the variations could be attributed to the large waveform fluctuations at each individual site, some other location-to-location variations could not. Synchronized local clusters coupled by gap junctions were also observed in the slices from adult rat hippocampus; the size of such clusters was less than 200 μm and oscillated at ~200 Hz (Draguhn et al., 1998).

This large location-to-location variation was not seen in our optical recordings. Comparing optical imaging and local field potential recordings, we noticed that the waveform fluctuations in the optical signals were substantially smaller than in LFP signals from the same area. The waveform variation in optical signals at different locations was also small. The comparison of LFP and optical signals suggests that the fluctuations in the activity only occurred at the local level. The signal on each optical detector was proportional to an averaged population activity in a volume of $330 \times 330 \times 400 \mu\text{m}^3$ of cortical tissue. In contrast, the tungsten LFP electrodes may mostly only measure the multidirectional current flowing in and out of the tip point. Thus globally a dynamic ensemble appears as an area of moderate depolarization with an overall low population firing rate. Within this area of moderate depolarization, there may be many localized neuronal clusters with different activity patterns.

Spiking activity within a dynamic ensemble

Tungsten extracellular microelectrodes were used for measuring the spiking activity in dynamic ensembles. In four preparations, 6600 spikes from 240 dynamic ensembles (evoked at an interval of 30 seconds) were recorded by a microelectrode placed in cortical layer II. The spiking density during dynamic ensembles was not high; there were only 28 ± 6 spikes during each ensemble (duration of $300 \pm **$ ms). Because the spiking density was sparse, and the amplitude of the spikes was distributed around 30 ± 4 distinguishable groups, we estimated that these spikes came from more than 30 neurons near the electrode tip. With this measurement we estimate that on average, each neuron fired ~ 1 spike during each ensemble, suggesting that neurons were moderately depolarized with a low rate of spiking. In the same set of data we did not find consistent spiking correlations in a millisecond time domain, although in the 100 ms time domain (about $1/4 - 1/2$ of the duration of each dynamic ensemble), the spiking correlation was high. This further suggests that the spiking of the neurons was not tightly synchronized even though they were physically located around the electrode tip.

Network excitability and the dynamic ensemble

The excitability of the cortical network was crucial for evoking dynamic ensembles. When the network excitability was altered, the optimized stimulation often failed to evoke dynamic ensembles. When the excitability was elevated by a small increase in $[K^+]$ (from 2 mM to 3 mM) or reduction of $[Mg^{++}]$ (from 2 mM to 1 mM) in the perfusing media, the probability for an optimized stimulation to evoke dynamic ensembles was substantially reduced (tested in three preparations, the effect was reversible; data not shown). Slight suppression of the GABA_A receptor mediated network by perfusing a very low dose of bicuculline (0.05-0.2 μ M) could completely block the evoking of dynamic ensembles by an optimum stimulation. At this concentration of bicuculline the inhibition in the network was only partially depressed (Chagnac-Amitai and Connors, 1989), so that the stimulation could not evoke epileptiform events; only the passive response could be seen (data not shown). Epileptiform events could be evoked when the preparation was perfused with a higher concentration of bicuculline (> 20 μ M). Epileptiform events had a significantly larger amplitude and propagation velocity (Tsau et al., 1998; Demir et al., 1999) than did dynamic ensembles.

A moderate decrease in excitability also substantially reduced the probability of evoking dynamic ensembles. An increase in $[Mg^{++}]$ from 2 mM to 3.5 mM in the bath to reduce NMDA receptor activity blocked the evoking of dynamic ensembles ($N=3$ preparations, about 100 trials each). This is consistent with the results of using NMDA and/or AMPA antagonists APV and CNQX (Metherate and Cruikshank, 1999). Computational models also suggest that NMDA receptor channels are important for sustaining the activity of neuronal ensembles (Lisman et al., 1998). Hsieh et al. (1998) found that the cholinergic agonist carbachol also has a strong effect, even at a very low dose (5 μ M), and can block dynamic ensembles. These results suggest that dynamic ensembles are sensitive to the balance of excitation-inhibition in the intrinsic cortical network. However, since a dynamic ensemble can only be evoked by an optimized stimulation condition, our experiments do not rule out the possibility that in an altered excitability dynamic ensembles can be evoked using other stimulation parameters. Although at each altered condition we confirmed that a dynamic ensemble could not be evoked using a large range of stimulation intensities, we did not attempt an exhaustive search for other methods which could evoke dynamic ensembles at each set of altered conditions.

In contrast to the manipulations mentioned above, the metabotropic glutamate receptor antagonist MCPG, even at a very high concentration (tested at 100 μ M on 3 preparations), did not block dynamic ensembles. This suggests that the dynamic ensemble is different from the network events mediated by metabotropic glutamate receptors (Whittington et al., 1995; Traub et al., 1996).

PROJECT 2: INITIATION OF EPILEPTIFORM ACTIVITY IN NEOCORTEX

In a number of epilepsy models, epileptiform activity can be initiated from cortical structures (McNamara 1994; Traub et al. 1994), indicating that the initiation of epileptiform activity can be intrinsic to the cortex. Studying the process of epilepsy initiation has been attractive not only because the understanding of its mechanism would benefit more efficacious prevention and treatment of the disease, but also because the study of neuronal interactions during this process would reveal important features of the functional neuronal organization in the cortex. Pyramidal neurons in layer V of the neocortex have been thought to be responsible for initiation, because pyramidal neurons have the highest excitability in the neocortex (Connors 1984), they have little detectable inhibition (Chagnac-Amitai and Connors 1989b), and layer V alone is sufficient for generating epileptiform activity in a zero Mg^{2+} epilepsy model (Silva et al. 1991). However, it has recently been reported that isolated superficial layers can also initiate epileptiform activity and may even dominate the initiation process in the intact cortex perfused with 10-20 mM bicuculline methiodide (Albowitz and Kuhnt 1995). Field potential recordings also suggested that epileptiform activity could start in layer II/III of the cortex (de Curtis et al. 1994). Since intrinsic firing properties of cortical neurons may underlie the initiation of epileptiform activity (Prince 1967, 1969; Traub and Wong 1982; Miles and Wong 1983), cortical neurons with repetitive firing properties might be capable of initiating epileptiform activity. This implies that various neurons from different layers may potentially become initiation cells for epileptiform activity if they possess repetitive and rhythmic firing properties. In fact, cortical neurons in both superficial and deep layers can be induced to fire spontaneously (Flint and Connors 1996), and therefore these neurons should potentially be able to initiate epileptiform activity. The location of initiation cells for triggering epileptiform activity in different cortical layers indicates the involvement of different neuron types in the formation of the epileptiform focus. In order to clarify in which layer the initiating cells are located, it is necessary to simultaneously monitor neuronal activity in all cortical layers during epileptiform activity.

Examination of the spatial and temporal characteristics of epileptiform activity in cortical slice preparations using conventional electrophysiological techniques requires an electrode array. The number of electrodes in such an array has been limited to just a few (Chagnac-Amitai and Connors 1989b) because of practical difficulties. Current source density analysis has been attempted in a study of the origin of epileptiform activity, but the location of initiation site could not be determined accurately (de Curtis et al. 1994). The timing of epileptiform activity onset from the different regions is a good indicator for where the epileptiform activity starts, but can only be compared when these regions are simultaneously measured. Because this transient signal tends to be in the millisecond domain, such measurements need high temporal resolution.

Optical recording has been applied to achieve the measurement of rapid changes in transmembrane potential, such as action potentials. The optical signals from neuronal processes stained with voltage-sensitive dyes are linear with membrane potential (Ross et al. 1977). If multiple neurons

are recorded optically by a single detector, the optical signal represents the average of the change in transmembrane potential (Tsau et al. 1996). When multiple regions are simultaneously recorded by a photodiode array, the timing of activities in these regions can be compared and the initiation site for epileptiform activity can thus be determined. In this study, we applied high speed (1 frame per msec) optical imaging using voltage-sensitive dyes to measure epileptiform activity over a region of the cortex about 4.5×1.5 mm², in an attempt to directly record the initiation process. We have reported that dominant foci for initiating epileptiform activity emerge when the preparation is perfused with zero Mg²⁺ artificial cerebral spinal fluid (ACSF) or normal ACSF containing bicuculline (20-50 mM) (Tsau et al. 1998). In this report we image these stationary foci in neocortical tissue. We ask in which cortical layer these foci are located. We also use electrical stimulation via a microelectrode to trigger epileptiform activity in various cortical layers to visualize the initiation process.

Spontaneous epileptiform activity initiation sites revealed by optical recordings

Spontaneous epileptiform activity emerged 20-40 min after the preparation was perfused with zero Mg²⁺ ACSF. Epileptiform events occurred in episodic bursts, each burst starting with a large initial spike and followed by 7 to 10 Hz oscillations lasting 5-30 sec (Silva et al. 1991; Flint and Connors 1996). We have validated the optical recordings by comparing simultaneously recorded electrical and optical signals throughout all experiments (n=46). These results indicate a very good correspondence between electrical and optical signals. Both recordings show a large initial spike followed by prolonged 7 to 10 Hz oscillations. The timing of the initial spike and some peaks of the oscillation waves was very similar in the two traces. However, details of the two recordings were not the same, probably because electrical recordings can be affected by current from distant areas, while optical recordings strictly reflect the neuronal membrane potential changes in the area whose image is projected to the recording photodiode (Salzberg et al. 1973; Ross et al. 1977). For example, the electrical recording is very sensitive to a stimulus current delivered at a substantial distance, while the optical signal is not affected at all. Therefore, it was anticipated that the components of field potential recording would differ somewhat from those of the optical recording. In this study, we were mainly interested in the timing and location of the initial spike in different cortical laminae in order to understand the initiation process of epileptiform activity. Because the timing of the initial spike in optical recordings correlated well with that of the local field potential recordings, we can use the photodiode array to locate the starting focus of the initial spike. In the following text we will only describe the characteristics of the initial spike of each epileptiform event. The secondary oscillations have different characteristics and have been described elsewhere (Wu et al. 1999).

Epileptiform activity could be recorded from the entire preparation when the preparation was perfused with zero Mg²⁺ ACSF (n=40), even in a small section (2 mm wide horizontally) of a cortical slice with all cortical layers intact. This was true of neocortical slices from randomly chosen regions including frontal, temporal, and occipital areas. This result indicates that initiation sites for generating epileptiform activity can be spontaneously formed regardless of the region of cortex. The initiation site in each stained cortical slice was optically identified and moved to an appropriate place in the imaging field. Because the resolution of optical imaging data is relatively poor compared to the cortical architecture due to the limited number of pixels, more than one cortical layer was often projected to a single detector and distinctions between layers could not always be made based on the imaging data. The onset of the epileptiform event started earliest in the trace recorded from the middle layer and was delayed (by about 10 msec) in the adjacent detectors. The further the physical location was from the

initiating site, the more delayed the onset. Consecutive image displays showed that the activation of cortical neurons appeared in a middle layer and then expanded to the superficial and deep layers as well as in lateral directions. These results indicated that epileptiform activity was initiated by an epileptiform focus located in layer III or IV in the parietal cortex. The location of such a focus was quite stable; repeated optical recordings ($n=10$) from the same preparation revealed the same initiation site and propagation profile. This is consistent with the finding that there often exists a dominant epileptiform focus (Tsau et al. 1998).

In some preparations, the initiation sites were found to be located in deep layers (probably layer V or VI). The epileptiform events started from deep layers and spread out to the adjacent areas. In other preparations, the initiation sites were found to be in superficial layers (probably layer I or II). Optical recordings were repeated 4 to 10 times on each preparation. These initiation sites were stable, and shifting of the initiation site was not observed. Therefore, it seemed that spontaneous epileptiform activity could be initiated in any of the cortical layers. All of the imaging data on the epileptiform focus showed that the activation started from a small confined area less than $0.3 \times 0.3 \times 0.4$ mm, or 0.04 mm³, and spread smoothly from the initiation site to adjacent cortical areas.

The initiation site across the cortical layers varied from preparation to preparation. These sites were scattered over the slice and were observed in neocortical slices from occipital, parietal, temporal, and perirhinal areas in different preparations ($n=11$). Among the eleven preparations, 5 had initiation sites in deep layers, 2 in middle layers, and 4 in superficial layers. These initiation sites seemed to be randomly distributed and not to have higher susceptibility in any particular cortical region. Although evidence has been presented that the temporal cortex may be more likely to originate epilepsy (e.g. Wardas et al. 1990), our data suggests that there is no preference for a particular cortical area or an exclusive cortical layer in establishment of an initiation site for epileptiform activity when the cortex is hyperexcitable and capable of conducting traveling epileptiform events.

Continuous recordings of spontaneous firing of cortical neurons

Our optical imaging data have shown the dynamic process of epileptiform activity initiation. In all the experiments, no consistent firing of cortical neurons was optically observed before an epileptiform event was initiated. No consistent firing was optically observed even at the initiation site. Since each photo-detector covers 0.04 mm³ of cortical tissue, significant firing of a single neuron may be masked by light noise from many other cortical cells. Another limitation of optical imaging is that each trial of recording is limited to a few seconds due to dye bleaching. We used a microelectrode in many locations in the slices in order to detect constant firing of neurons. This allowed us to record spikes from the neurons close to the recording electrode's tip. In most experiments (68 out of 72), no continuous firing was observed. Occasionally (4 experiments), continuous firing from a single neuron was observed before as well as during epileptiform events. However, none of the activity was correlated to the initiation of epileptiform events. These data indicate that spikes in a single cortical neuron may not be able to initiate epileptiform activity. Although the recording period can be as long as several hours, the major limitation of the electrical recording is that it is very difficult to accurately position the electrode at the initiation site of an epileptiform event.

Electrically evoked epileptiform events

We showed above that spontaneous epileptiform events can be initiated in several cortical layers; it seems that cortical neurons in several layers have this capability. However, it is possible that an initiation site that appears in middle or superficial layers is actually initiated by layer V pyramidal neurons whose discharges trigger the activation of an initiation site located in one of the other cortical layers. Optical recordings might not be able to detect a small initiating discharge from layer V pyramidal neurons. This implies that even though we have seen the activity starting from the superficial or middle layers, it might actually be triggered by undetectable activity in the deep layers, and neurons in superficial layers might not be capable of initiating epileptiform activity themselves. To test this possibility, a microelectrode was used to measure the ability of an electrical stimulus to initiate epileptiform events in different layers. A single electrical pulse (10V 0.1 msec) was delivered via an electrode (its tip diameter was less than 1 mm, usually about 0.1 mm) with impedance of 2-5 MW, placed in one of the cortical layers. Assuming that the electrical impedance of fluid and brain tissue is much lower than that of the microelectrode and can be ignored, the stimulation current $I = V/R$ would be 2-5 mA at the electrode's tip. The current density would be 2500 times smaller at a distance of 50 mm from the tip, on the assumption of current spread from a point source in a volume conductor. Therefore, only the neurons close to the tip would be directly activated by the stimulus, and the number of neurons should be very limited. We found that epileptiform events were reliably evoked in all cortical layers by the electrode stimulation. The evoked epileptiform events had the same characteristics as those of spontaneous events, and propagated vertically and horizontally. The initiation site depended only on the position of the stimulation electrode's tip. The images, made at 2 msec intervals, show clearly that activation first appeared at the electrode tip with no delay and then spread smoothly to the neighboring areas. No jumping of activation from the electrode tip to deep layers or other cortical areas was observed in any of the experiments. Various areas of the cortex including frontal, occipital, parietal, temporal, perirhinal, and hippocampal cortex were tested; all the cortical structures were found to be capable of generating evoked epileptiform events ($n=36$). Since the timing for stimulation was arbitrarily set, the evoked epileptiform events must have started independently of any spontaneous pyramidal neuron discharges.

DISCUSSION

Our optical imaging data directly revealed spontaneous initiation sites. These initiation sites were found to be stable in every preparation. In different preparations, the initiation sites were in different cortical layers where different types of neurons are located. Continuous spontaneous activity correlated with epileptiform events was not observed in either the focus or other regions between epileptiform events. We also used extracellular microelectrodes to monitor single unit activity during as well as between epileptiform events and did not observe consistently firing neurons in the neocortical slices. Therefore, it is reasonable to assume that most neurons, except those responsible for initiation, are not continuously firing between epileptiform events. We have previously hypothesized that a dominant initiation focus may be organized by a dynamic process in a group of local neurons (Tsau et al. 1998); our current results suggest that this kind of process is not laminar-specific, and it can happen in various cortical layers.

Optical signals reflect the membrane potential change in all membranes stained with voltage-sensitive dyes. Because neurons contribute to the optical signal regardless of their physical size, a change in membrane potential of virtually all the cortical neurons could contribute to the optical signals. As we report in this study, the initiation site can be located in several cortical layers, and so would be composed of neurons of different types since each cortical layer has distinctive cell types. Different types of neurons in various layers may become initiation neurons and trigger epileptiform activity.

Previous studies showed that elimination of layer V abolishes spontaneous activity in cortical slices (Silva et al. 1991). In order to explain our data of visualizing initiation foci in superficial layers, one may assume that an epileptiform event may be triggered by an initiation site located in layer V pyramidal neurons, but the activities in layer V are too small to be detected by our optical recording technique. We used the microstimulus experiment to reduce this possibility. When epileptiform events were evoked by electrical stimulation, the number of neurons activated directly by stimulation was presumably limited. In this experiment we always saw the activity start from the stimulation point instead of from layer V. This suggests that an epileptiform event starts at the layers where the stimulation electrode is placed. The current density of stimulation is high around the microelectrode's tip and the effective area is probably smaller than a distance of 50 μm . This volume of cortical tissue can contain only 100 neurons with a 10 μm diameter. It is therefore likely that the neurons directly activated by the stimulation electrode also start the epileptiform activity. However, our data does not rule out the possibility that layer V pyramidal neurons provide background activity for the neurons in other layers; certain patterns of the spontaneous activities in layer V may trigger other layers to start all-or-none epileptiform events. It is difficult for our current experimental setting to demonstrate that pyramidal neurons provide background activities. However, Demir et al. (1999) suggested similar activities, a "pre-epileptiform activity" which can trigger an all-or-none epileptiform event in other places. Cortical neurons in both superficial and deep layers in the neocortex can have repetitive discharge properties (Flint and Connors 1996). Neurons in isolated superficial layers of entorhinal cortex can also fire repetitively (Dickson and Alonso 1997). It has long been believed that neurons with repetitive discharge properties are capable of initiating epileptiform activity (Prince 1967, 1969; Traub and Wong 1982; Miles and Wong 1983). Our data further suggests that neurons in various layers may be capable of initiating epileptiform activity, and that the initiation process may be less reliant on one specific type of neuron.

The initiation site revealed by our optical imaging data was composed of a confined volume of less than 0.04 mm^3 , indicating that the initiation of epileptiform events is a localized process and may be from local neuronal clusters with potentiated connections (Tsau et al. 1998). As this is the smallest volume a photodiode could detect in our experiments, the real epileptiform focus could be smaller. We hypothesize that a neuron pool with repetitive discharge properties in such a confined epileptiform focus may synchronously activate its adjacent neurons and thus initiate epileptiform activity. This is consistent with the stimulation experiment where a single pulse stimulus generates synchronized activation of cortical neurons around the microelectrode tip and results in an all-or-none epileptiform event.

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COMPUTATIONAL NEUROSCIENCE

GEOFFREY GOODHILL, PH.D.

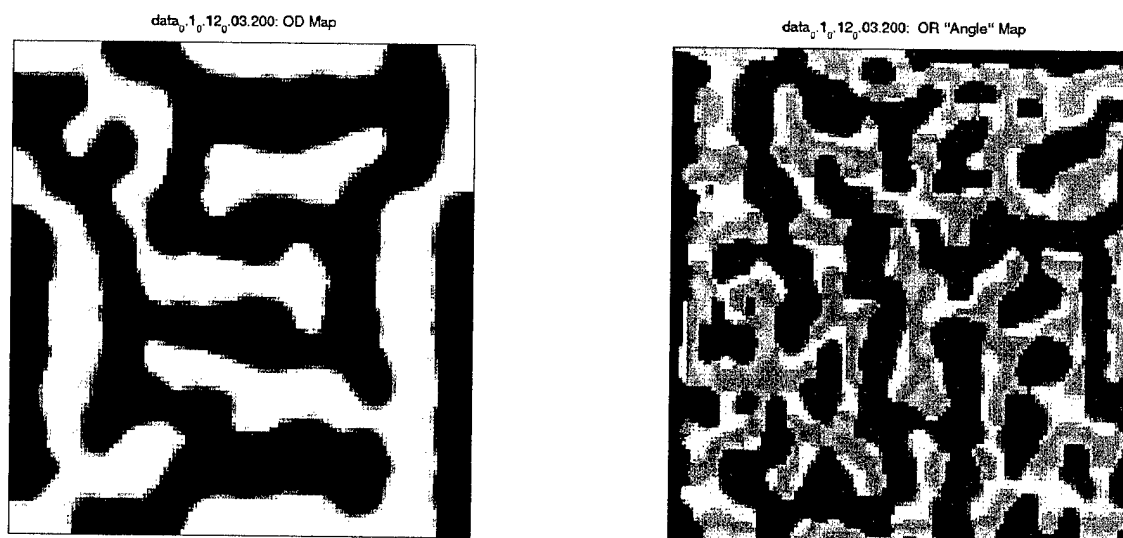
Dr. Goodhill has two main research goals, both broadly relating to neural development. The first goal is to understand the theoretical principles governing the development and structure of cortical mappings. The second research goal is to understand quantitatively how gradients of chemotropic factors guide axons to appropriate targets in the developing brain.

Project 1: Development of Cortical Maps

Two key questions for understanding sensory processing are, how is information about the world represented in the cortex, and how are these representations formed during development. The best studied cortical areas in this regard are V1 and V2, where features of the visual scene such as position, orientation, direction and spatial frequency are represented in maps. Understanding how these maps and the relationships between them come about is an important step in answering the questions posed above. The relative importance of genetic versus epigenetic (particularly activity-dependent) mechanisms in determining cortical structure is still a subject of intense debate. This debate impacts directly on the design of effective therapies for treating the effects of early sensory visual experience.

Dr. Goodhill has been testing the hypothesis that cortical map structure arises from activity-dependent learning rules that attempt to represent highly correlated input features close together, acting in conjunction with genetically determined constraints such as the shape of the target area. To do this he has been using the elastic net algorithm, a theoretical model of cortical development that is well suited to exploring questions of large-scale map structure. Fig. 1 shows results of the algorithm applied to a five dimensional feature space: two dimensions represent spatial position, one represents ocular dominance, and two represent orientation preference θ coded as two cartesian dimensions ($\cos\theta$, $\sin\theta$). Extending the mathematical analysis of Durbin et al (1989) he has derived expressions for how the bifurcation points of this algorithm vary with the parameters, corresponding to when each columnar system forms. By varying the parameters it is therefore possible to allow ocular dominance columns to develop first (Fig. 1a) or orientation columns to form first (Fig. 1b). An interesting difference in the structure of the ocular dominance column map is apparent between these two cases. If ocular dominance columns develop first then they have a fairly regular structure with a well-defined periodicity. If on the other hand orientation columns develop first then the structure of the ocular dominance column map is less regular. It is possible that this explains the differences seen in maps between cats and monkeys; he is now working to characterize more quantitatively the precise differences between these maps. Over the past year this work was partly supported by an NSF grant IBN-9808364 awarded to Dr. Goodhill entitled "The Development and Structure of Visual Cortical Maps"; for the next four years it will be wholly supported by an NIH Grant 1R01EY12544 awarded to Dr. Goodhill with the same title.

A



C

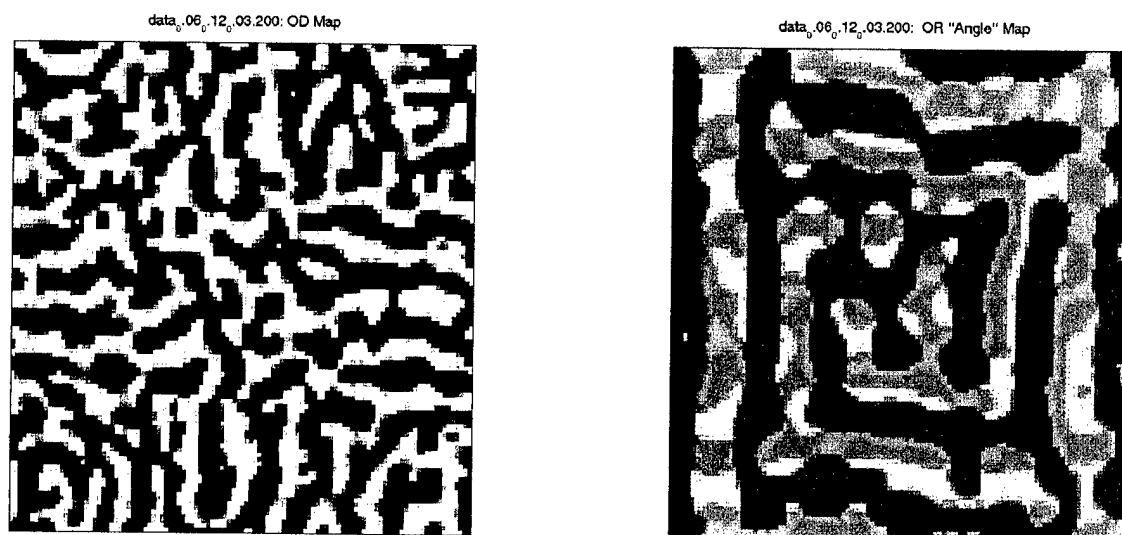


Figure 1: Ocular dominance and orientation maps produced by the elastic net algorithm. For the orientation maps, each color represents a particular orientation in a regular progression between 0 and 180 degrees. Note the presence of "pinwheels", where all orientations are represented around a point. **A** Ocular dominance map develops first. **B** Orientation map develops first. Note the very different appearance of the ocular dominance map in these two cases.

PROJECT 2: AXON GUIDANCE BY GRADIENTS

Axon guidance by gradients plays an important role in wiring up the developing nervous system. Growth cones sense a concentration difference between their two ends, and convert this into a signal to move up or down a gradient. Previously, Dr. Goodhill formulated a very simple mathematical framework to understand when and where gradient detection can occur as a function of gradient shape. This framework was applied to two examples: the guidance of axons by target-derived diffusible factors *in vivo* and in collagen gels, and to guidance by substrate-bound gradients of optimal shape, as might be relevant in the retinotectal system. More recently, in collaboration with Dr. Jeffrey Urbach (Physics Dept.), Dr. Goodhill investigated a much more sophisticated model of gradient sensing (originally developed by Berg & Purcell (1977)) and derived its predictions for growth cones. The model predicts that the minimum detectable gradient steepness for a diffusible ligand in three dimensions is about 1%, in good agreement with the (extremely crude) experimental data that currently exists. However, the model also predicts that this value should be much higher, about 10%, for detection of a substrate bound factor (due to the lower ligand diffusion rate). This predicted difference remains to be investigated experimentally. On June 1, 1999, Dr. Goodhill submitted an NIH grant 1R01NS39955 entitled "Theoretical Analysis of Axon Guidance" which proposes to develop this work further.

In addition to this theoretical work, Dr. Goodhill (again in collaboration with Dr. Urbach) is also developing a novel experimental system for testing hypotheses about the mechanisms of axonal gradient detection. Using cutting-edge nanotechnology, they have built a device to "paint" a pattern of varying concentration of a guidance molecule onto the surface of a long, thin block of collagen gel. The molecules then diffuse into the collagen, creating a gradient. Numerical calculations and preliminary data show that the concentration will quickly become uniform across the (small) thickness of the block, but that a stable gradient, reflecting the original pattern on the surface, will exist along the length of the block for several days (Fig. 2). Axons can then be grown in gradients of different shapes, and their behavior analyzed as a function of parameters such as gradient steepness. This will yield precise, quantitative data to constrain models of the mechanisms of gradient sensing by growth cones. Two model systems are currently being investigated: the guidance of dorsal root ganglion axons in response to Nerve Growth Factor, and the guidance of cortical axons in response to netrin-1. Since no fluid culture medium can be present in this assay an important piece of preliminary data is to show that axons can be successfully grown in "dry" collagen: we have now achieved this (Fig. 2). This work was partly supported by an NIH Shannon Award 1R55RR13342 awarded to Dr. Goodhill entitled "Precisely Controlled Gradients for Axon Guidance", and for the next two years will be partly supported by an NIH grant 1R21NS39354 with the same title.

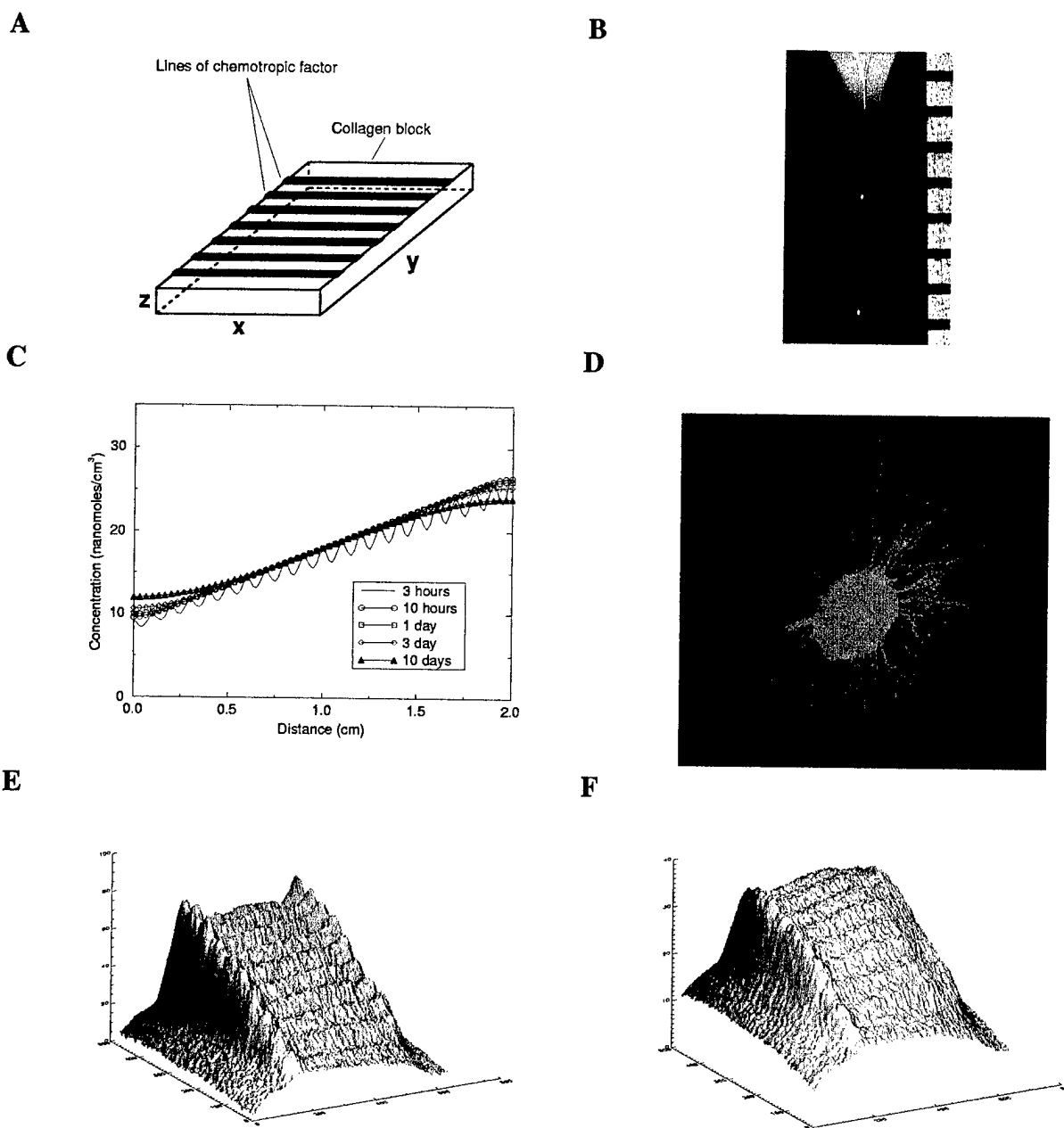


Figure 2: Results for the gradient assay. **A**. Basic method for establishing the gradient. **B**. Instantaneous image of a stream of droplets ejected from the pump, captured with strobe illumination: the drops are of volume 1 nl, and the ruler spacing is 1 mm. **C**. Simulation result showing that, after the initial transients have died away, the gradient (y axis) along the length of the block (x axis) remains highly stable (linear gradient initially consisting of 21 lines 1 mm apart; other gradient shapes have similar stability properties). **D**. Fluorescence image of TUJ-1 stained P3 rat dorsal root ganglion grown for three days in block of collagen with no fluid medium. **E**. Measured gradient of fluorescent dye (casein) in a section of a collagen block 30 minutes after generation of the initial pattern. **F**. Same gradient after 4 days. Note that gradient shape remains highly stable. Total extent of gradient along x, y axes is 1 cm (axis units are pixels), z axis units are arbitrary units of fluorescence intensity (separately normalized for E, F).

DRUG DISCOVERY AND DESIGN: Dr. Kozikowski's work relates to the design and synthesis of new pharmacological research tools for understanding brain mechanisms, including cognitive drug development. Dr. Wang utilizes molecular modeling techniques as part of drug development, as well as studies relating to structural biology.

ALAN P. KOZIKOWSKI, Ph.D.

TRH Analogues for Traumatic the Treatment of Neurodegenerative Diseases

To date we have prepared a number of analogues of thyrotropin-releasing hormone (TRH) that may find use in traumatic brain injury, stroke, Alzheimer's disease, and other neurodegenerative conditions. Recent work that involves extensive collaborations between the research groups of Dr. Kozikowski and Dr. Faden has shown that two classes of compounds which may be viewed as distant analogs of TRH show superb neuroprotective properties. Two prototype molecules, which are the subject of two recently filed GU patents, are exemplified by the tripeptide disulfide **I** and the cyclohexyl bearing diketopiperazine **II** (Figure 1). Neither of these molecules show much affinity for the TRH receptors per se, and thus their mechanism of action is unlikely to involve direct interaction with these receptors. Interestingly, compound **I** contains the chemically reactive disulfide linkage, which is likely to be converted in vivo to its monomeric mercaptan. Therefore, in analogy to the mechanism of action of glutathione,¹ we believe that the neuroprotective properties of **I** may be associated with its free radical scavenging properties. As such, we believe that it is likely that additional novel structures can be created by combining the remote TRH-like mimicry of these compounds with another pharmacological action known to be linked to neuroprotection. Therefore, with the aid of continued funding from the DOD moneys, it is our plan to synthesize other structures that are likely to disrupt radical damage inducing cascades, those involving both reactive oxygen intermediates and nitric oxide. As such our design rationale involves building additional novel diketopiperazine and tripeptide structures in which nitric oxide synthase inhibitors and oxygen radical traps are embedded. Tables 1 and 2 provide structures for the new compounds to be created.

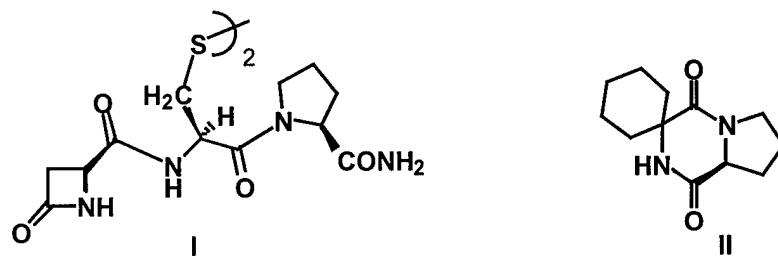
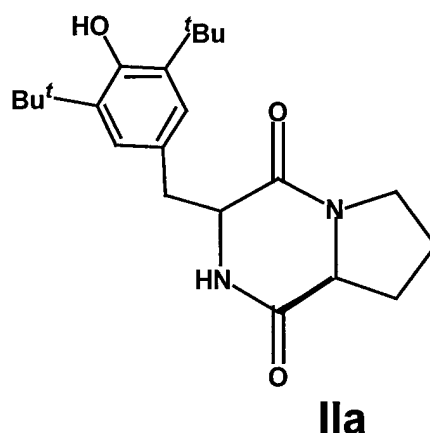


Figure 1. Structures of Neuroprotective Tripeptide **I** and Diketopiperazine **II**

Reactive oxygen intermediates include the superoxide radical anion, hydrogen peroxide, and the very aggressive hydroxyl radical. The free radical mediated oxidation of cellular macromolecules (lipids, proteins, DNA, etc.) has been implicated in a number of disease states, including stroke and head trauma.² These radicals, acting mainly through the initiation of chain reactions, can cause extensive damage to unsaturated lipids found in neuronal membranes, thereby resulting in neuronal cell death and

consequent neurological impairment. Thus, one novel approach to the development of neuroprotective agents has been based upon the discovery of molecules that act as free radical traps that are capable of intercepting chain initiators and/or chain carriers. Small molecular weight antioxidants already present in the body which are able to act in this manner include glutathione, thioredoxin, and vitamins C, and E.¹

Thus, within the context of further exploring TRH-like structures possessing an antioxidant action, we have synthesized a molecule that incorporates a free radical trap into the diketopiperazine structure. Specifically, we have synthesized structures like IIa that incorporate the di-*t*-butyl catechol moiety. This compound is now under extensive study by the Faden laboratory, and significant neuroprotective effects are found with this compound under conditions inducing radical damage (Fenton type chemistry). Additional scale up of larger quantities of this novel neuroprotective agent are now underway, and further experiments including in vivo studies are planned.



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Design of Inhibitors of CPP32.

Apoptosis derives from a Greek word that means to fall off, as leaves from a tree. It is a process in which the cell shrinks, and eventually the apoptotic cell bodies that are produced are phagocytosed. Apoptosis is characterized by preservation of membrane integrity, cytoplasmic and nuclear condensation, reduction in cellular volume, plasma membrane bleb formation, and nuclear fragmentation. During apoptosis the morphological changes are often accompanied by internucleosomal cleavage of genomic DNA; the breakdown of the DNA occurs in discrete fragments of 180-200 base

pairs, which appear as a DNA ladder in agarose gel electrophoresis. Apoptosis differs from necrosis in that the latter involves cell death that is accompanied by inflammatory processes. Thus in apoptosis cells can die one at a time, among a group of healthy cells, whereas in necrosis a whole group of cells may die at the same time. Death by necrosis is generally associated with a traumatic injury, ischemia, chemical exposure, and the like. Apoptosis is often confused or mixed up with programmed cell death (PCD). The latter term originated to describe cell death occurring at normal stages of the developmental program and that is initiated by some physiological trigger. In some cases PCD of cells shares morphological changes that are similar to those of apoptosis, but this is not always so.

While a complete pathway for cell death has not been defined, it is very clear from a number of studies that the CED-3/ICE family of proteases plays an essential role in apoptosis. The CED-3 homologue, CPP32, also known as apopain, plays a definitive role in cell death, for it is involved in the destruction of certain key homeostatic and repair enzymes at the onset of apoptosis. CPP32 degrades proteins by effecting cleavage of functional domains comprised of the (P₄)Asp-X-X-Asp(P₁) motifs. Targets for destruction by CPP32 include poly(ADP-ribose) polymerase (PARP, which is an enzyme involved in DNA repair), the 460,000 M_r catalytic subunit of the DNA-dependent kinase essential for double-strand break repair, the 70000 M_r small nuclear ribonucleoprotein necessary for mRNA splicing, and other enzymes including protein kinase C delta. Studies have revealed that levels of CPP32 are elevated during apoptosis, and that its removal from apoptotic cellular extracts can lead to a reduction in apoptosis in an *in vitro* type of assay.

Of particular interest to our own research is the structural work that has been done on apopain. Researchers at the Merck Research Laboratories have recently been able to determine the three-dimensional structure of apopain in complex with the tetrapeptide aldehyde inhibitor, Ac-DEVD-CHO.^{1,2} This inhibitor has been shown to have a 49-fold preference for CPP32 versus ICE (interleukin-1 β converting enzyme, which belongs to a second phylogenetic subfamily of proteases, and whose role in apoptosis is less secure). In contrast, the tetrapeptide aldehyde Ac-YVAD-CHO is far more selective for ICE (K_i = 0.76 nM) than for CPP32 (K_i = 10,000 nM).

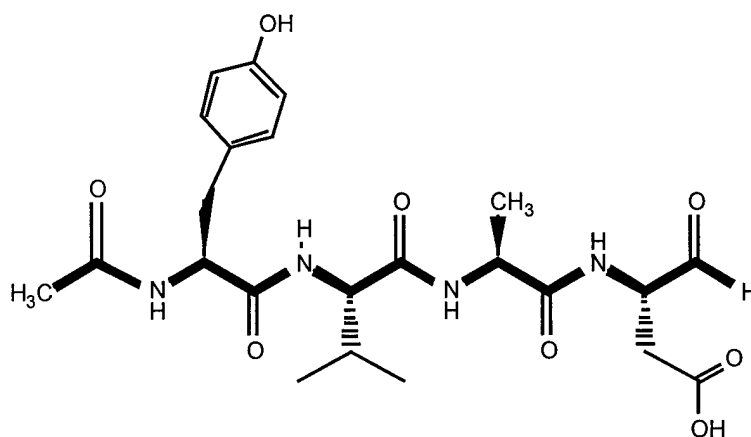


Figure 1. Ac-YVAD-CHO, selective inhibitor of ICE

The x-ray work reveals that the tertiary and quaternary structure of these cysteine proteases are similar. In both proteases two heterodimers associate to form a tetramer, a four-chain assembly with two-fold rotational symmetry. A diagram of the tetrapeptide aldehyde inhibitor bound to CPP32 is provided below. As is clear from this diagram, Cys 285 engages in thiohemiacetal formation with the aldehyde group, thus mimicking the transition state for amide bond hydrolysis. The binding pockets of

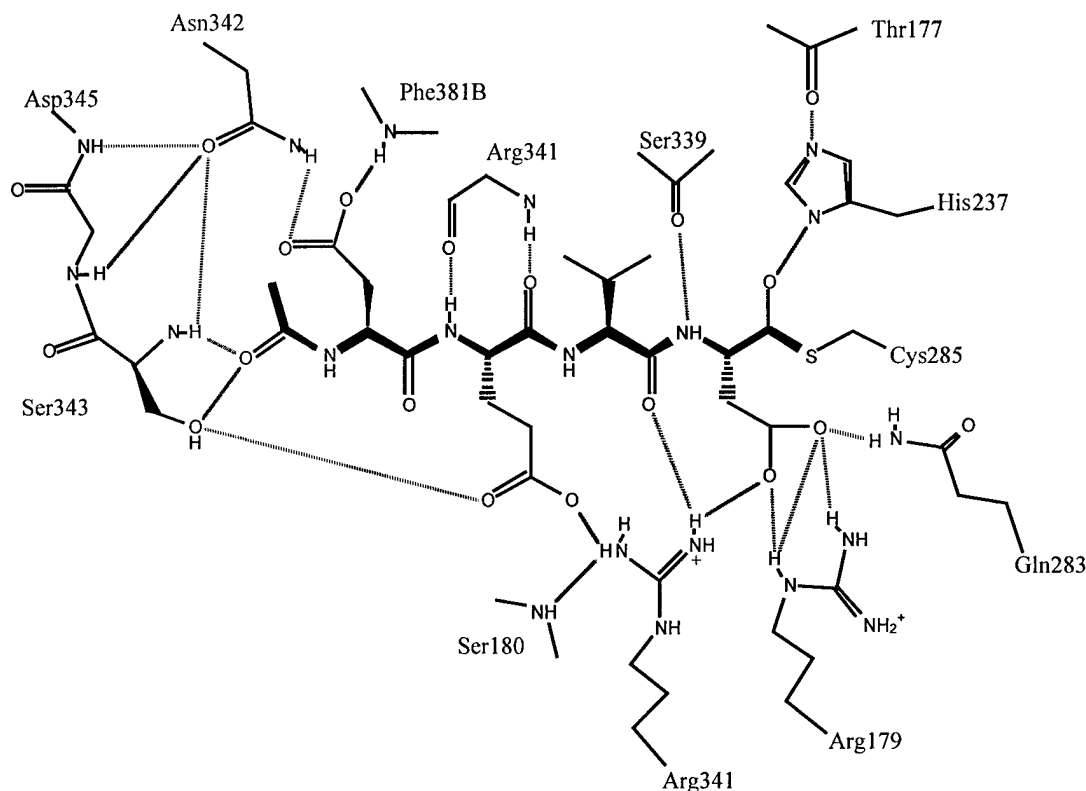
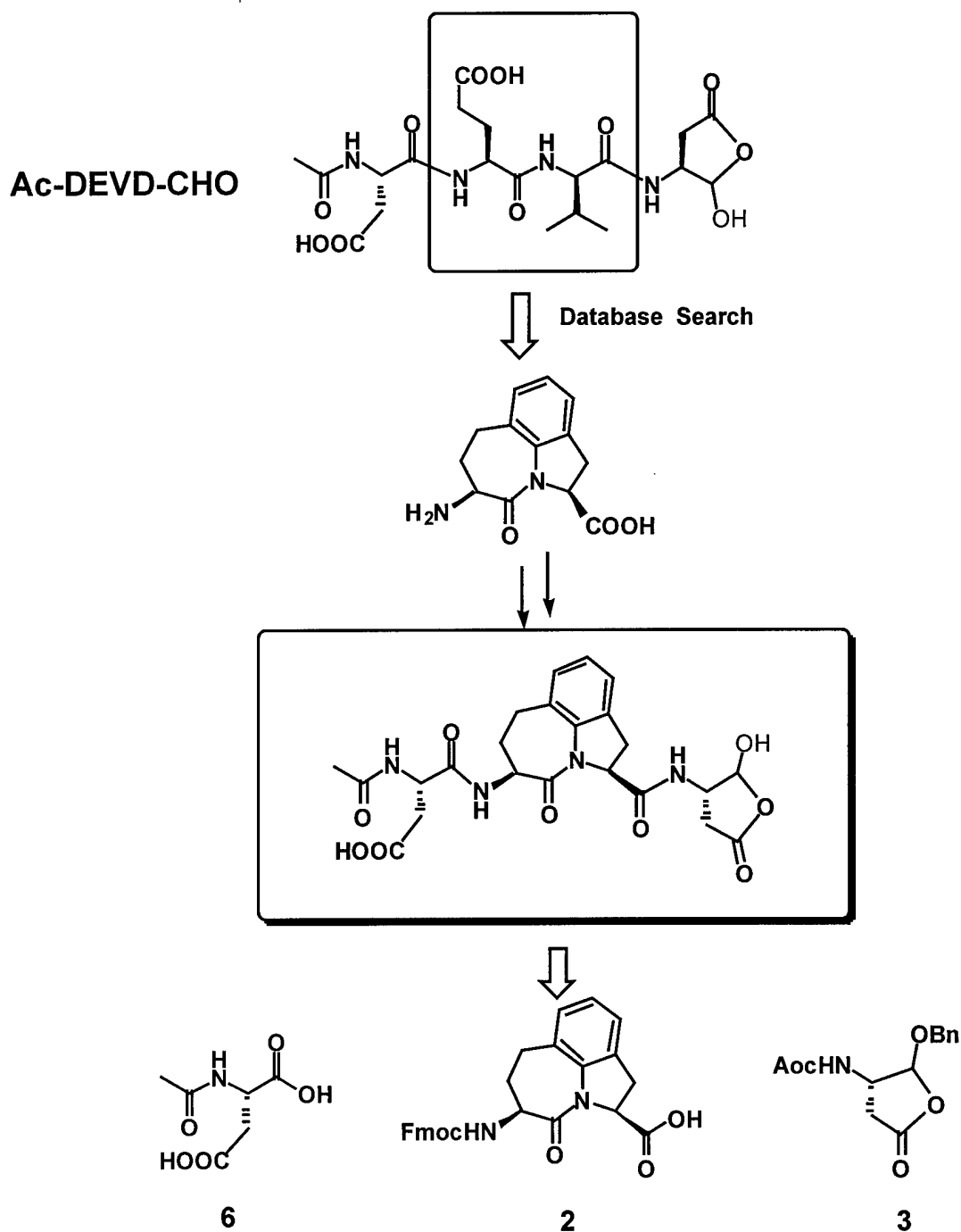


Figure 2. Hydrogen bonds and other polar interactions between the bound tetrapeptide aldehyde inhibitor and CPP32/apopain.

ICE and apopain are similar, with the exception particularly of the S₄ subsite which appears to be responsible for the differences in substrate specificities. As expected from the preference for a P₄ Tyr in the ICE inhibitors, the corresponding subsite in the ICE enzyme corresponds to a large, shallow hydrophobic depression, while the P₄ site in apopain is narrow and intimately engulfs the P₄ aspartate residue side chain. Time dependent irreversible inhibitors of ICE have been designed that are acyloxymethylketones, and which react by displacement of the carboxylate group by the active site cysteine 285.

In order to discover compounds that may serve as cell permeable and selective inhibitors of these cysteine proteases, and in particular CPP32, we have chosen to investigate approaches based upon database screening methods in which a pharmacophore query is set up employing various elements of the x-ray structural information of the tetrapeptide inhibitor Ac-DEVD-CHO in complex with CPP32. Using this information, several very weak lead compounds were identified by Dr. Wang, and this information has been used in conjunction with rational drug design concepts to devise new inhibitors of CPP32. To date, Dr. Nan working in the laboratory of Dr. Kozikowski has synthesized

approximately 15 molecules that have been tested for their inhibitory activity in the laboratory of Dr. Faden. One of these molecules (structure shown below) which was prepared using a combination of data base screening to identify a mimic of the dipeptide EV together with drug design concepts was found to be a very potent inhibitor of Caspase-3, with a K_i of ~ 3.5 nM. This compound was less active at Caspases-7 and -8 (K_i s ~ 722 nM and 433 nM, respectively), while its activity at Caspase-1 was about 50 nM. This molecule still contains a reactive aldehyde group, and we are currently exploring whether this must be retained to achieve compound potency.



We are now in the process of designing other caspase inhibitors based upon this lead structure. It is our aim to achieve the design of selective inhibitors of CPP32 that: 1) contain no hydrolyzable peptide bonds, 2) are cell membrane permeable, and 3) are capable of functioning without the need for a "reactive" aldehyde group. This project represents a significant new area of our research program, and one which we believe holds tremendous promise in the search for new therapeutics in the treatment of major brain disorders including stroke and Alzheimer's disease.

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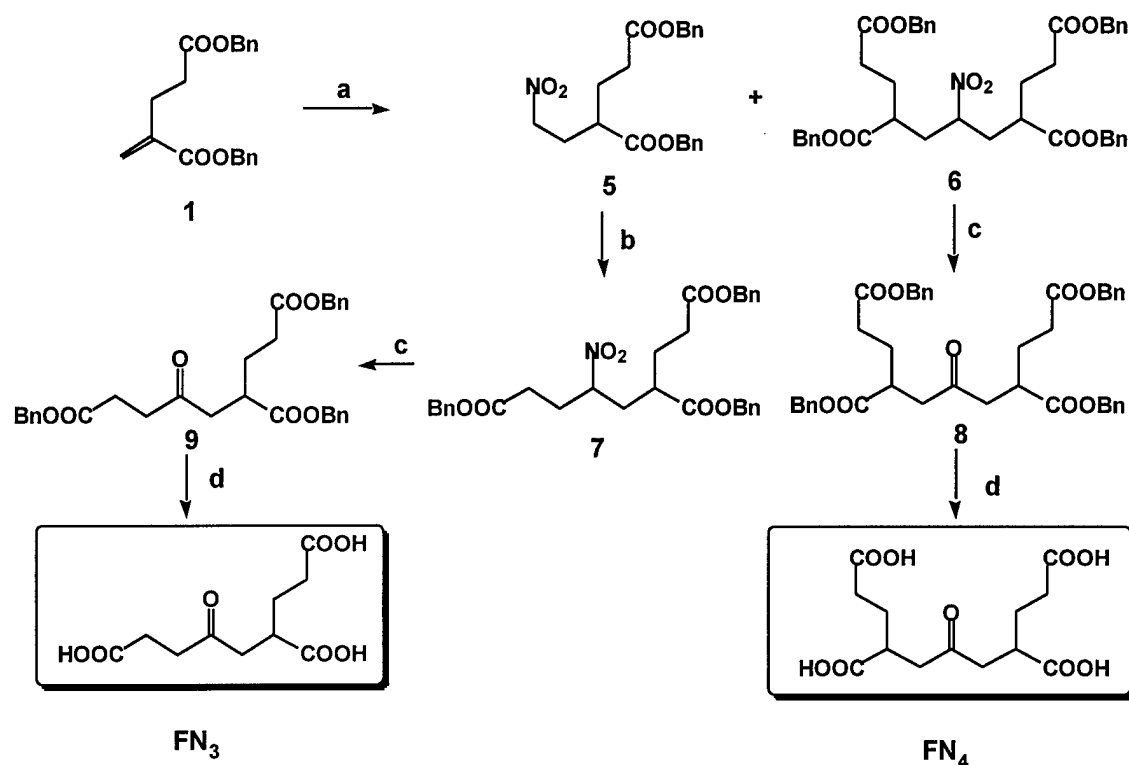
Dual Function Glutamate-Related Ligand: Discovery of a Novel, Potent Inhibitor of Glutamate Carboxypeptidase II and a Selective mGluR3- Agonist

The metabotropic glutamate receptors (mGluRs) are a heterogeneous family of G-protein linked receptors that couple to multiple second messengers. These include the negative modulation of adenylate cyclase, activation of phosphoinositide-specific phospholipase C, and modulation of ion channel currents.¹ Three types of mGlu receptors have been identified: group I receptors couple to phosphoinositide hydrolysis and include mGluR1 and mGluR5; group II receptors are coupled to inhibition of cyclic adenosine 5'-monophosphate(cAMP) formation and include mGluR2 and mGluR3; group III receptors(mGluR4, mGluR6, mGluR7 and mGluR8) are negatively coupled to cAMP.² Each subtype is thus distinguished on the basis of its pharmacology and sequence homology. Excessive activation of glutamate receptors or disturbances in the cellular mechanisms that protect against the potential adverse consequences of physiological glutamate receptor activation have been implicated in the pathogenesis of a diverse group of neurological disorders. These disorders include epilepsy, ischemia, central nervous system trauma, neuropathic pain, and chronic neurodegenerative diseases. Because of the ubiquitous distribution of glutamatergic synapses, mGluRs have the potential to participate in a wide variety of functions of the CNS. In addition, because of the wide diversity and heterogeneous distribution of mGluR subtypes, the opportunity for the development of highly selective drugs that affect a limited number of CNS functions exists. The mGluRs therefore provide novel targets for the development of therapeutic agents that could have a dramatic impact on treatment of CNS disorders.

To date, almost all of the commonly used agonists and antagonists employed in biological studies of the mGluRs are amino acids, often embodying a structurally rigidified glutamate-like core.³ During our efforts to identify potent and selective ligands acting at these receptors, we have discovered an mGluR3 selective agonist that contains only acid groups.

The starting point of our studies is N-acetyl-L-aspartate-L-glutamate (NAAG), which is a peptide neurotransmitter that is widely distributed in the mammalian nervous system.⁴ This peptide is inactivated by an extracellular peptidase activity(NAAG peptidase or glutamate carboxypeptidase II) producing glutamate and N-acetylaspargate (Figure 1).⁵ The peptidase is concentrated, if not exclusively localized, in glia. NAAG is a low potency agonist and may act as a partial antagonist at some NMDA receptors.⁶ In studies using cell lines transfected with mGluR1-6, NAAG was found to selectively

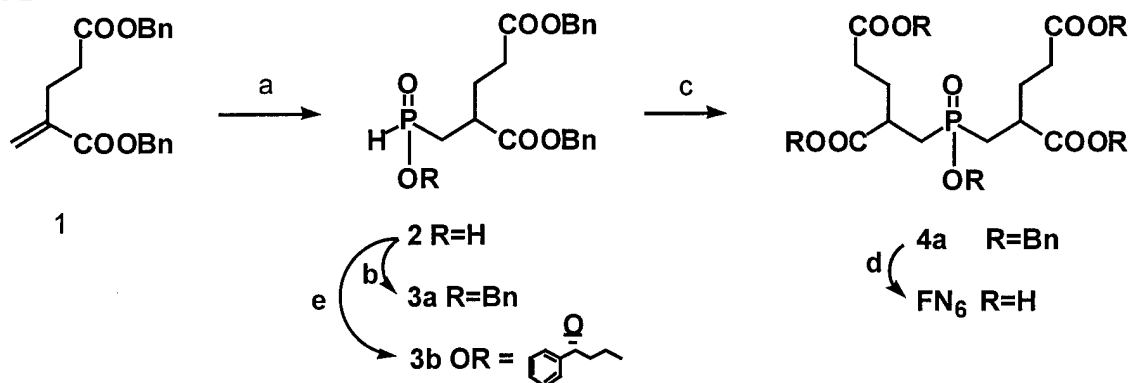




Reagents and conditions: (a) CH₃NO₂, Triton B, rt, 24 h; (b) Triton B, benzyl acrylate, CH₂Cl₂, rt, 24 h; (c) CTAP, Et₃N, CH₂Cl₂, rt, 4 h; (d) 20% Pd(OH)₂/C, H₂(1atm), rt, 12 h.

The synthesis of FN₃ and FN₄ are outlined in **Scheme 1**. Compound **1** was prepared using a literature method.⁸ Conjugate addition of nitromethane to compound **1** in the presence of Triton B afforded a mixture of compound **5** and **6** in a ratio of 2:1.5. After separation, compound **5** underwent another conjugate addition to benzyl acrylate to afford compound **7**. Employing the same procedures, compound **6** and **7** were converted to carbonyl compound **8** and **9**, respectively, which were finally transformed to compound FN₃ and FN₄ after hydrogenolysis. The synthesis of FN₆ is outlined in scheme 2. Compound **1** was prepared using a literature method⁸ Conjugate addition of Sodium hypophosphite to α-methylene dibenzyl glutarate **1** afforded the phosphonite **2**, which was converted to benzyl group protected compound **3a** in presence of trimethylacetyl chloride.¹⁰ Deprotonation of compound **3a** with NaH followed by conjugate addition to compound **1** provided the all protected pentaester **4a**, which was finally transformed to compound FN₆ after hydrogenolysis (**Scheme 2**).

Scheme 2



Reagents and conditions: (a) NaH_2PO_2 , TMSCl , Et_3N , then compound **1**, rt, 24 h; (b) PivCl , BnOH , $\text{CH}_2\text{Cl}_2/\text{py}$ (10 : 1), rt, 2 h; (c) NaH , THF , 0°C , then compound **1**, rt, 2 h; (d) 20% $\text{Pd}(\text{OH})_2/\text{C}$, 70 psi H_2 , rt, 24h, (e) PivCl , $\text{R-(+)-1-phenyl-1-butanol}$, $\text{CH}_2\text{Cl}_2/\text{py}$ (10 : 1), rt, 2 h.

As the above synthesis proceeds in a stereorandom fashion, we prepared also the three optically pure isomers of **FN₆** through the use of the chiral alcohol $\text{R-(+)-1-phenyl-1-butanol}$ (see Scheme).

Compound **FN₆** and its three stereoisomers were tested for both inhibition of NAAG peptidase and mGluR activity. **FN₆** showed potent inhibition of NAAG peptidase with an IC_{50} of 4.4 nM. It also showed high selectivity to mGluR3 compared to other members of metabotropic glutamate receptor family (**Table 1**). The individual stereoisomers did not show significant differences compared to the isomeric mixture **FN₆**, and apparently, stereochemical requirements are negligible. Hence the absolute stereochemistry of the three isomers was not determined.

Table 1 EC_{50} Values for **FN₆ and its stereoisomers to NAAG peptidase and mGluR3**

Compound	NAALADase Inhibition (EC_{50} , nM)	mGluR ₃ Agonism (EC_{50} μM)
FN ₄		
FN ₆	4.4	4.5
FN ₆ -1	4.8	
FN ₆ -2	10	
FN ₆ -3	3.6	
FN ₆ -4	4.7	

In summary, we describe the discovery of a dual acting ligand that offers a novel approach for the generation of possible neuroprotective agents for the treatment of neurodegenerative disorders. As this molecule FN₆ acts both as an mGluR3 selective agonist, and as a potent inhibitor of the enzyme involved in the cleavage of NAAG to glutamate, it is simultaneously able to control levels of glutamate and to activate an mGluR subtypes known to confer neuroprotection. The mGluR selectivity of this new type compound may make it a useful pharmacological agent for resolving mGluR₃ function at identified synapses. Further efforts aimed at improving compound potency as well as selectivity are in progress, as are the study of the neuroprotective effects of this compound in cell toxicity models. Additionally as the NAG peptidase appears to be associated with certain types of cancers such as prostate, the effects of this compound on cell proliferation are being examined.

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SHAOMENG WANG, Ph.D.

Dr. Wang is interested in computational structural biology (protein folding, drug and receptor interactions), design and discovery of novel therapeutic agents for neuroprotection.

PROJECT 1: PROTEIN FOLDING AND PROTEIN MIS-FOLDING

INTRODUCTION:

Three-dimensional structures of enzymes ultimately determine their biological properties. As genome science rapidly evolves and develops, it is increasingly important to have the ability to predict the three-dimensional structures of enzymes of interest since experimental determination of the 3D-structures of enzymes of interest using either X-ray diffraction or multi-dimensional NMR techniques is painstaking and slow. Toward this end, we have developed a novel and powerful computational method termed "self-guided molecular dynamics simulation method" and applied this method to the protein-folding problem for the studies of the dynamics, thermodynamics and kinetic properties of protein folding and the prediction of protein structure.

METHODS AND RESULTS:

Among many prediction and search approaches, molecular simulation method displays a number of promising prospects. It takes atomic interaction details into account and directly incorporates both enthalpy and entropy effects. However, molecular simulation of a large system such as enzymes with thousand atoms is very time consuming and the time scale it can access is very limited (often limited to nano-seconds). Many important biological processes such as protein folding and drug-receptor binding takes place in a time scale of from micro- to milliseconds and there are beyond the reach of current computer power with conventional simulation techniques. Therefore, in order to study these biological processes using simulation methods, we have to either simplify the systems of interest or to improve the simulation efficiency dramatically.

We have developed a new algorithm called "self-guided molecular dynamics simulation approach", which has been shown to improve the conformational searching efficiency by 100- to 1000-folds. This algorithm was presented in the Gordon Research Conference in Computational Chemistry in June 1998 and was cited by many researchers as the most exciting paper in the Gordon Conference. A paper described this algorithm has been published in *Journal of Physical Chemistry, B.* (1998, 102, 7238-7250) and in *Journal of Chemical Physics* (1999, 110, 9401-9410). This new method provides us with the opportunity to investigate one of the most challenging and difficult problems in modern biological science, i.e. the protein-folding problem. Since October 1, 1998, we have applied this method to the folding of peptides and proteins with defined secondary and tertiary structures and have made significant progress towards the protein-folding problem.

First, we have studied the folding of protein secondary structure because Protein structures are composed of elementary secondary structures, including turns, α -helices and β -sheets. The formation of these secondary structures is believed to play an important role in protein folding, which remains to be one of the major unsolved and most challenging problems in modern biological science. An in-depth understanding of folding of these basic secondary structures is an important step toward solving the

protein-folding problem. Short peptides (<20 residues), which adopt significant populations of secondary structures in solution are excellent model systems with which to study folding of protein secondary structures.

Up to date, we have successfully simulated the folding of peptides with turns, helices or β -sheets. Our studies have begun to provide important insight into the folding of protein secondary structures in a number of important aspects including the folding mechanism, factor that govern the folded structure and folding dynamics. One manuscript on the folding of a type II turn structure has been submitted for publication in the Journal of the American Chemical Society and another paper concerning the folding of α -helix in aqueous solution has been submitted to Journal of Molecular Biology.

Since October 1, 1998, we have started to investigate the folding of proteins with defined tertiary structures. To date, we have successfully simulated the folding of a 23-residue small protein designed to mimic the zinc finger binding protein. In addition, we have successfully simulated a 33-residue protein with 3-helices and a 56-residue protein with 4-helices. Two manuscripts on the folding of these two proteins are being prepared. Collectively, these studies will provide important clues to the protein-folding problem.

FUTURE DIRECTIONS:

Since this new algorithm improve the simulation efficiency in an order of 100- to 1000-fold in comparison with convention simulation methods, we will be able to apply this efficient and novel new algorithm to fold peptides with up to 50 amino acids to investigate the detailed protein folding mechanism such as β -amyloid, which has important implication in Alzheimer's disease. We will also be interested to utilize our new method in protein structure prediction. Using the information obtained from the protein folding studies, we will be able to design small molecule drugs that can block the misfolding of amyloid from α -form to β -form.

PROJECT 2. DESIGN AND DISCOVERY OF CASPASE-3 SPECIFIC INHIBITORS

INTRODUCTION:

Apoptosis, or programmed cell death (PCD), is triggered by a number PCD factors. Inappropriate apoptosis is now believed to contribute to the pathology of several human diseases, including neurological diseases such as Alzheimer's disease and Parkinson's disease, and solid tumors. A number of genes have been identified to play key roles in apoptosis. In *C. elegans*, these genes include CED-9, CED-4, CED-3. In addition, two additional factors, cytochrome C (also designated as Apaf-2, apoptotic protease activation factor 2) and dATP play crucial roles in the activation of CED-3. It has been shown that CED-9, which functions upstream of CED-3 and CED-4, negatively regulates the apoptotic program by preventing the activation of CED-3 and CED-4, probably through blocking the release of cytochrome C, a key activation factor for CED-3, from mitochondria. CED-4 is required for the activation of CED-3, in addition to cytochrome C.

The apoptotic program delineated in *C. elegans* is conserved in mammalian cells. The corresponding homologs to CED-9, CED-4 and CED-3 are BCL-2, Apaf-1, and caspase-3. Bcl-2 can block the release of cytochrome C from mitochondria, which prevents the activation of caspase -3 (caspase was derived from cysteine aspartase for these cysteine proteases always cleave a site with aspartate residue on the C-terminal). In the presence of cytochrome C and dATP, Apaf-1 binds to cytochrome C and activates caspase-3, although the precise mechanism of this activation is not known.

The activated caspase-3 is capable of autocatalysis as well as cleaving and activating other members of the caspase family, leading to rapid and irreversible apoptosis. Activated caspase-3 will cleave and activate the DNA fragmentation factor, DFF, which in turn leads to the degradation of DNA into nucleosomal fragments, a hallmark of apoptosis. Deletion of caspase-3 from the mouse genome through homologous recombination results in excessive accumulation of neuronal cells, owing to a lack of apoptosis in the brain. Addition of active caspase-3 to normal cytosol activates the apoptotic program. A specific, high affinity ($K_i < 1$ nM) caspase-3-specific tetrapeptide inhibitor, Ac-DEVD-CHO, can abolish the ability of cytosol from apoptotic cells to induce apoptosis in normal nuclei and block the initiation of the cellular apoptotic program in response to apoptotic stimuli. These data clearly suggest that caspase-3 is both necessary and sufficient to trigger apoptosis. Although a tetra-peptide inhibitor, Ac-DEVD-CHO, is of high specificity and of high affinity and has served a useful role in defining the enzymology and function of some members of caspase-3 protease family, it is of limited utility for advanced drug development. Clearly, a high affinity, non-peptide, specific caspase-3 inhibitor is of great value to study the functions of caspase-3 in vivo systems. Unfortunately, non-peptide caspase-3 inhibitors have not been reported.

We are well poised for the structure-based discovery and design of non-peptide caspase-3 inhibitors since the X-ray structure of the tetra-peptide inhibitor Ac-DEVD-CHO in complex with caspase-3 has been determined. Based upon the X-ray structure, the cysteine 285 sulfur reacts with the aldehyde group on the tetra-peptide inhibitor and forms a thiohemiacetal, which is stabilized by His 237 through a hydrogen bond formed between the hydroxyl of the thiohemiacetal group and the nitrogen at the d1 position on the His 237 ring. The carboxylic group of the aspartic residue at P1 position plays an important role by forming a number of hydrogen bonds with Arg 179, Gln 283 and Arg 341. The hydrophobic side chain of valine residue at P2 position in the inhibitor interacts with a number of hydrophobic residues, including Tyr 338, Trp 340, and Phe 381. The glutamate residue at P3 position forms a number of hydrogen bonds with the side chain of Arg 341 and Ser 343. The aspartic acid at P4 position forms two hydrogen bonds between its carboxylic group and the side chain of Asn 342, and with the backbone NH group of Phe 381. The carbonyl group of the acetyl group forms a strong hydrogen bond with the backbone NH group of Ser 343.

RESULTS AND DISCUSSION:

Based upon the X-ray structure of caspase-3 in complex with the tetra-peptide Ac-DEVD-CHO, the aldehyde group is essential for the inhibitor to react with the sulfur group of Cys 285. In addition, the carboxylic group of the aspartate residue at the P1 position on the inhibitor plays a crucial role through interacting with a highly charged positive pocket in the receptor binding site. The isopropyl side chain of the valine residue at the P2 position on the inhibitor appears to be of importance since it interacts with a number of hydrophobic residues in the receptor. As mentioned above, the glutamate residue at the P3 position and the aspartate residue at the P4 position may be important for both binding

affinity and specificity. Attempts to identify compounds that will mimic all these important interactions were not successful. Thus, we turned our efforts to identify compounds that will mimic the crucial interactions at the P1 and P2 positions. We have therefore constructed a pharmacophore model that consists of the aldehyde group and the carboxylic group on aspartate residue. The geometric parameters between these two groups, as revealed by the X-ray structure, were incorporated into the pharmacophore model since these parameters are probably important for the effective interactions between the inhibitor and the receptor. A 3D-database search of the National Cancer Institute 3D-database of 216,000 compounds retrieved a number of compounds that meet the requirements of the pharmacophore model. The samples of these compounds were obtained and these compounds were then evaluated for their ability to block the caspase-3-like activity in cellular extract in collaboration with Dr. Alan Faden. One compound with molecular weight less than 300, displays significant activity (we estimated that its activity is about 500-fold less potent than the extremely potent tetra-peptide inhibitor, Ac-DEVD-CHO). Based upon this compound, we have designed new compounds that can mimic all the essential moieties in the tetra-peptide inhibitor.

Using the information obtained from the X-ray structure and our molecular modeling, we have been able to design a class of non-peptide inhibitors. In collaboration with Dr. Alan Kozikowski, we have synthesized the first inhibitor within this new chemical class. The biochemical testing was performed in Dr. Faden's laboratory. It was found that this new class of non-peptide inhibitor is more potent than the Ac-DEVD-CHO tetra-peptide inhibitor. Additional compounds based upon the novel leads have been designed and are being synthesized now in Dr. Kozikowski's lab. As soon as we complete our synthesis, we will test these compounds in Dr. Faden's lab, first their enzymatic activity, then their neuro-protective activity.

FUTURE DIRECTIONS:

Over the last year, we have demonstrated that our approach is very effective in the design of potent and selective CPP32 inhibitors. In fact, our designed compound has a superior potency than the Ac-DEVD-CHO tetra-peptide inhibitor. Additional new compounds have been designed and are being synthesized in Dr. Kozikowski's lab. It is expected that our multi-disciplinary team will be able to discover novel neuro-protective agents targeting this important apoptotic enzyme.

PROJECT 3: DEVELOPING NEW MOLECULAR DOCKING METHODS FOR STRUCTURE-BASED DRUG DESIGN (MCDOCK AND Q-JUMPING).

INTRODUCTION:

There are now more than 7000 three-dimensional (3D) coordinates of proteins or nucleic acids determined by X-ray crystallography or nuclear magnetic resonance (NMR) spectroscopy available from the Protein Data Bank (PDB, 1998) and the total number of the 3D structures is increasing with even greater speed every year. Many of these macromolecules serve as potential therapeutic targets and the availability of 3D structures of these macromolecules offers us unprecedented opportunities for structure-based drug design and discovery. To effectively carry out structure-based drug design, two fundamental questions need to be addressed. The first question is how a drug (or ligand) fits into the receptor binding site (the docking problem) and the second question is how well it binds to its receptor

(the binding affinity prediction problem). We have developed two new docking methods, Monte Carlo Docking (MCDOCK) and q-jumping methods to address the docking problem.

METHODS AND RESULTS:

In the MCDOCK, receptor and ligand 3D structures are used as the input data. Often the 3D-structure of the receptor (enzyme) is obtained through X-ray diffraction or NMR techniques, or computer predictions. The structure of the ligand can be generated from computer programs such as CORINA. Then, ligand is placed into the binding pocket of receptor by comparing the relative orientation of them. This step is called geometry-based docking, which serves as a pre-docking. This step is quick but not very accurate. Although in some cases, it can predict the binding-mode of a ligand quite well. After the pre-docking step, energy-based MCDOCK is performed to search potential energy minimums by combining different Markov chains and different sampling techniques. There are two kinds of Markov Chains employed in the energy based docking, single main chain and multiple side chains. Each point along these chains represents a random sampled configuration. We split the searching task into non-localized and localized searching, which are performed in the main chain and side chains respectively. The main chain searching is assigned with a higher temperature in the canonical sampling. This is a non-localized searching and the probability to overcome certain energy barrier is increased with increasing temperature. Larger step lengths are employed in the main chain searching. Consequently, it is less precise to locate the accurate local minimum in the potential surface. This main chain is divided into several segments and the configuration with the lowest energy within each segment is recorded. Those side chains are propagated from these recorded configurations in main chain by a simulated annealing method, which is to gradually reduce the temperature in the MC simulation and more precisely locate local minimum. The step lengths in the side chain simulations are normally set to be about 10 to 50% of that in the main chain simulations. The side chain searching is a localized searching. The main purpose of the side chain simulations is to more precisely locate the local minimum. The lowest energy configuration from these side chains is then further refined by increasing interaction cut-off value. We also implemented different sampling schemes to optimize the probability to overcome different energy barrier, such as global sampling of three overall Euler's angles and all torsion angles of ligand as well as Markov chain sampling of all variables. To enhance the searching efficiency, there is one importance sampling distribution developed in our Monte Carlo searching using an effective temperature.

We have tested 29 complexes which their binding modes are known from X-ray diffraction. For the rigid body docking, an average RMS (root of mean square) value of 0.5 Å was obtained for all-non hydrogen atoms for the ligand between the predicted and the X-ray determined binding modes. For flexible ligand docking, RMS of 1 Å was obtained. In addition, our method is very fast. On average, it takes about 60 seconds for rigid body docking and less than 900 seconds for flexible ligand docking. Therefore, this new method will be a powerful tool for the studies of drug-receptor interactions and for structure-based drug design and discovery. A paper describing the MCDOCK method has been published in *Journal of Computer-Aided Molecular Design* (1999, 13, 435-451).

Receptor flexibility has been a very difficult and challenging problem in docking. Towards this goal, we have developed new features in the MCDOCK program to include the receptor/enzyme flexibility into account. A paper describing this significant progress has been accepted for publication in *Journal of Computer-Aided Molecular Design*.

At the same time, we have developed another novel method for molecular docking based upon generalized entropy theory. We called this method as q-jumping. A paper describing this new method has been published in Journal of Chemical Physics (1999, 111, 4359-4361). Application of this new method to more docking problems has been very successful and a paper describing the results has been submitted to Journal of Physical Chemistry.

FUTURE DIRECTION

We plan to continue our investigation to include the enzyme/receptor flexibility into the docking studies. We have shown in the past year that this is an achievable goal. In addition, since water molecules play an important role, we plan to include water molecules in our docking studies. Significant progress has been made in this area in the last few months.

PROJECT 4: DESIGN AND DISCOVERY OF NOVEL THYROTROPIN-RELEASING HORMONE

INTRODUCTION:

In recent years, rational drug design has been proven to be a powerful approach to accelerate the new drug lead discovery and development, in comparison to conventional drug design approaches. The rational drug design approach can generally be divided into two major categories. The first category is the so-called receptor structure-based discovery design. The use of this powerful approach has been exemplified by the discovery and development of new anti-HIV drugs, HIV-1 protease inhibitors. The extensive use of the receptor-based drug design methods made the new anti-HIV drugs available on the market in seven years. The applicability of the receptor-based drug design approach is however limited to the availability of the three-dimensional (3D) structure of the receptor and the detailed characterization of its active site. In case where the 3D structure of the receptor of interest and/or its active site is not known, the second category of rational drug design approach, i.e. the ligand structure-based drug design approach, may be used. The basic idea of the ligand structure-based drug design is that the biological activity of a ligand, substrate, or inhibitor may be represented by a 3D-pharmacophore model. A pharmacophore model is defined as the representation of crucial chemical structural features and their 3D geometric relationships that are important for the biological activity of interest. The big advantage of the ligand structure-based, or the pharmacophore-based approach is that it does not require the availability of the 3D structure of the receptor. The only requirement is the availability of extensive structure-activity relationship (SAR) data. The other advantage of this approach is that the biological activity does not need to be the binding affinity to a specific receptor *per se*. The disadvantage of this approach is that the pharmacophore model(s) derived from this approach should be carefully validated through the use of known experimental data. Its predictive power also needs to be tested through the design, chemical synthesis and biological evaluation of new compounds. Over the years, Dr. Wang and his collaborators have shown that this approach is a very effective approach in both the discovery of new lead compounds and the optimization of leads.

A number of tripeptides and dipeptides, including TRH and of its related analogues, have been synthesized and evaluated in the Laboratories of Dr. Faden and Dr. Kozikowski as potential neuro-protective agents. Over the years, we have accumulated a large body of SAR data. Indeed, we are now very well poised to use these SAR data to develop plausible pharmacophore model(s) using the

molecular modeling techniques. These pharmacophore models will be first used to identify novel compounds that meet the pharmacophore model(s) through the 3D-database pharmacophore searching. These new compounds will be quickly screened using the assay developed in Dr. Faden's laboratory. These new experimental data will provide crucial experimental validations of the pharmacophore model(s) used in the 3D-database pharmacophore search and new data for the refinement of the pharmacophore models. Upon the validation and refinement of the pharmacophore models, these models will be then used to aid the design and selection of novel synthetic targets that will be synthesized in Dr. Kozikowski's laboratory. These synthesized compounds will be tested in Dr. Faden's laboratory. The new data will be again used to further refine and validate the pharmacophore model(s). This interactive approach of pharmacophore model generation, database searching, biological testing, new compound design, biological testing will be an effective and efficient approach for the rapid development of neuroprotective agents.

METHODS AND RESULTS:

Eight TRH analogues which were shown to have robust neuroprotective effect in our rat model were used to generate pharmacophore models. 3D structures of these molecules were first built using Catalyst, and the structures were then minimized using empirical force field in Catalyst. The optimized structure for each molecule was used as the starting structure to generate multiple, low energy but distinct conformations using the conformational analysis module within Catalyst with polling method. In this study, conformations within 25 kcal/mol from the lowest energy conformation of each molecule were selected to ensure that the conformations are relevant to the biological activity and be able to cover a significant portion of the conformational space. The number of low energy conformations selected for each molecule range from 124 to 363. All these conformations were included for the generation of pharmacophore models.

Chemical features used in generating pharmacophore models include hydrogen-bond donor, hydrogen-bond acceptor and hydrophobic center. The common feature hypothesis method in Catalyst, was used to generate potential pharmacophore models. Multiple pharmacophore models were generated and validated.

One pharmacophore, contains one hydrogen-bond donor, and two hydrogen-bond acceptors. The relative orientation between these two spheres determines the directionality of the hydrogen bond. This model was validated first with the eight active compounds used in the pharmacophore generation to examine if in fact each compound contains this pharmacophore model. The results are summarized in Table 1. It is clear that each of these 8 active compounds possesses the pharmacophore model as shown in Fig. 1, with a low energy conformation. For all the 8 compounds, the hydrogen bond donor that was mapped to the pharmacophore was the amino group of the terminal amide. The two hydrogen bond acceptors that were mapped to the pharmacophore model were the carbonyl group (C=O) in the pyroGlu site of the TRH and the nitrogen atom on the histidine ring that can function as a hydrogen bond acceptor for seven compounds. The only exception is for compound 3-ARA-57a.

Since the pharmacophore model was generated through the use of eight active compounds, the model should only be considered as the necessary but not the sufficient requirement for the activity within this class of compounds. To further validate the pharmacophore model, 7 inactive TRH analogues that were not used to generate the pharmacophore model were evaluated to investigate if they

also contain the pharmacophore model. The results are also summarized in Table 1. Three of them, 2-ARA-66b, 2-ARA-17b and 2-ARA-60a, could not be fitted into the pharmacophore model because of the lack of one crucial pharmacophore feature. Figure 3 shows the lack of the fit of 2-ARA-66b to the pharmacophore model. Two compounds, RX77368 and 2-ARA-29a, were able to fit into the pharmacophore model only when using relatively high energy conformations, compared to the 8 active compounds. Compound 1-ARA-98a could be fitted into the pharmacophore model. However, the mode mapped into the pharmacophore model was totally different from those for the active compounds. Of note is the inactive compound MK-771, which was fitted into the pharmacophore model very well with low energy conformation, in the same mode as those for active compounds. Thus, the pharmacophore model was able to successfully distinguish 14 out of a total of 15 compounds (8 actives and 7 inactives) but failed to explain the lack of the activity for one compound MK-771. This suggests that the pharmacophore model can be further improved and refined. In fact, we found that if one includes the substructure feature of ProNH₂ as the essential requirement for the activity, the expanded pharmacophore model can explain all active and inactive compounds. Based upon our experience, it is best to use a less stringent pharmacophore model when searching a database for the discovery of new lead compounds. However, it is better to use a more stringent pharmacophore model when designing new synthetic targets.

In summary, using the extensive SAR data, molecular modeling can be used to generate potential pharmacophore models that can be validated using experimental data. These validated pharmacophore models in turn will be used to search for large chemical database such as the National Cancer Institute 3D-Database and the Available Chemical Database of Molecular Design Limited to discover novel lead compounds. These pharmacophore models will also be used to guide the design and selection of new synthetic targets.

The Catalyst/DBServer was used to convert the NCI-3D database of approximately 200,000 compounds into the Catalyst 3D-database format. In addition, we have converted the Available Chemical Database (ACD) of 210,000 compounds, supplied from the Molecular Limited Inc. to the Catalyst 3D database format. The first pharmacophore model was then used to search these 2 databases. It was found that more than 100 compounds satisfy the pharmacophore requirements. Based upon the novelty of their chemical structures, we have evaluated the first 7 compounds in collaboration to assess their neuro-protection activity. It was found that one of such compounds has shown significant neuro-protection activity in our in vivo models. Thus, this non-peptide compound represent a novel and promising lead for further development.

FUTURE DIRECTION:

Additional analogs based upon the novel lead compound have been identified and are currently being tested in Dr. Faden's lab. It is expected that compounds with improved activity will be discovered. Furthermore, we will perform de novo design of novel compounds based upon our pharmacophore models. These compounds will then be synthesized in Dr. Kozikowski's lab and tested in Dr. Faden's lab. It is anticipated that our concerted efforts among 3 laboratories will yield novel therapeutic agents for neuro-protection.

MOLECULAR NEUROBIOLOGY AND PLASTICITY: Dr. Faden's group evaluates the molecular and cellular correlates of secondary neuronal injury, including apoptosis. Dr. Swope's research relates to the actions of novel receptor protein kinases.

ALAN I. FADEN, M.D.

Our laboratory studies the pathobiology of neuronal cell death associated with central nervous system injuries and examines both mechanisms of neuronal cell death, as well as development of novel pharmacological treatment strategies. The central underlying hypothesis being evaluated is that the initial insult (trauma or ischemia) initiates an endogenous autodestructive response, leading to delayed cell death through both necrosis and apoptosis. Elucidating the specific factors involved and their temporal profile can permit the development of drug approaches that serve to limit secondary injury, thereby improving outcome. Complementary *in vivo* and *in vitro* model systems are used to study the molecular and cellular biology of secondary cell death, as well as to examine the potential novel pharmacological strategies.

There are six major lines of investigation in the laboratory: 1) elucidating the role of caspases, and their regulatory pathways, in neuronal apoptosis and traumatic neuronal injury; 2) examining the modulation of polyADP-ribose polymerase (PARP) in neuronal trauma; 3) ceramide induced trauma cell death; 4) defining the role of the tumor suppressor gene product, PTEN, in neuronal apoptosis; 5) evaluating the role of metabotropic glutamate receptors (mGluR) in secondary injury; and 6) drug discovery related to neuroprotection and cognitive enhancement.

PROJECT #1: ROLE OF CASPASES IN NEURONAL APOPTOSIS AND TRAUMATIC NEURONAL INJURY

Our group was the first to establish an important role for the cysteine protease caspase-3 in posttraumatic cell death and neurological dysfunction, and amongst the first to demonstrate a role for this caspase in neuronal apoptosis. During the past year, we have utilized cerebellar granule cells subjected to trophic withdrawal to establish a specific role for caspase-3 in neuronal apoptosis. Cerebellar granules cells (CGCs) were co-transfected with a green fluorescent protein reporter and one of several hammerhead ribozymes constructed to cleave caspase-3 RNA. Use of such ribozymes is highly selective. In separate experiments we co-transfected with a gene that expressed the inhibitor of apoptosis protein (IAP or hILP), which is relatively selective for this caspase-3 family, as well as the baculovirus protein P35, which serves as a pan-caspase inhibitor. Each of these transfection experiments showed similar degrees of neuroprotection following trophic withdrawal, thereby establishing a role specifically for caspase-3 in apoptosis of neuronal cells (Fig. 1).

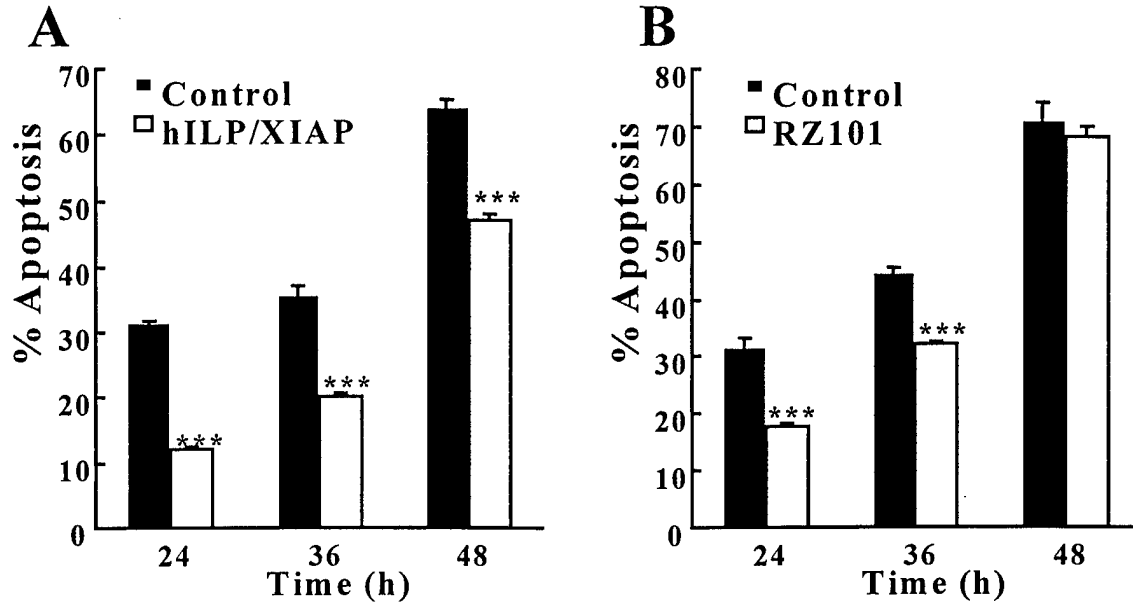


Figure 1A. Apoptosis in transfected CGCs expressing human ILP/XIAP. Apoptosis was induced by serum/ K^+ deprivation and cells were analyzed after 24, 36, or 48 h. Apoptosis in negative control cells expressing empty pEBB vector measured $32 \pm 0.3\%$ (average cells counted (x)=164), $36 \pm 1.3\%$ (x =171), and $64 \pm 1.1\%$ (x =79), respectively. In hILP/XIAP-expressing cells, apoptosis was $12 \pm 0.4\%$ (277), $20 \pm 0.6\%$ (185), and $47 \pm 1.1\%$ (103), respectively. *** $p < 0.001$ by one-tailed Student's t-test ($n=5$).

Figure 1B. Apoptosis in transfected CGCs expressing a ribozyme against rat caspase-3. Apoptosis was assessed after 24, 36, or 48 h of serum/ K^+ deprivation. In negative control cells expressing β -galactosidase, apoptosis measured $32 \pm 2\%$ (average cells counted (x)=69), $45 \pm 1\%$ (x =89), and $71 \pm 3\%$ (x =38), respectively. In cells expressing RZ101, apoptosis at the same times points was reduced to $18 \pm 0.7\%$ (x =158), $32 \pm 0.5\%$ (x =151), and $68 \pm 1.7\%$ (x =41), respectively. *** $p < 0.001$ by one-tailed Student's t-test ($n=5$).

Previously, we have demonstrated that caspases are expressed after traumatic brain injury in rats and that pretreatment with a caspase inhibitor, combined with post-treatment, significantly reduces neuronal cell death and enhances neurological recovery. In follow-up studies, we demonstrated that post-treatment with pan-caspase inhibitor zVAD, beginning 30 min after trauma, significantly improved both motor recovery and cognitive recovery (spatial learning and memory) following lateral fluid percussion head injury in rats (Fig. 2).

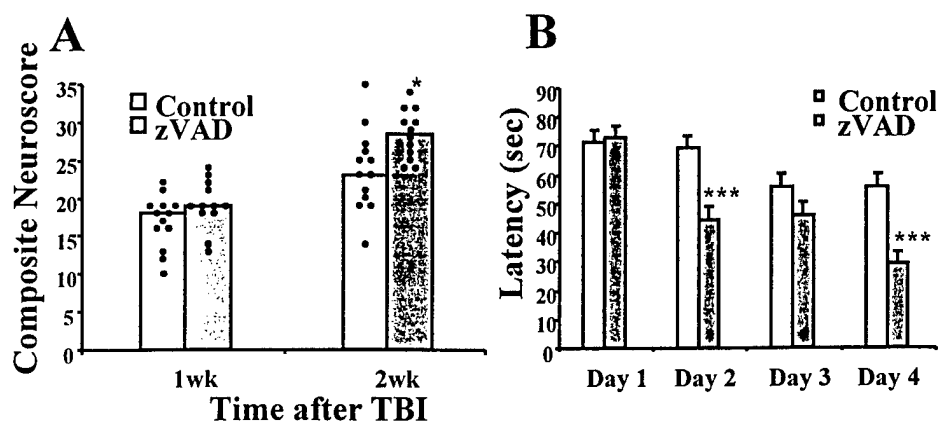


Figure 2. Recovery of neurological and cognitive function following fluid percussion injury in z-VAD or vehicle icv treated rats. (A) Columns represent median values for each treatment group whereas each dot represents an individual animal neurological score. At two weeks post-trauma, compound z-VAD treated rats showed significantly improved motor recovery. (B) Latency to find the hidden platform in a version of the Morris water maze. Results are expressed as daily means \pm SEM for each group over 4 trials. At two weeks posttrauma, z-VAD treated rats showed significantly improved spatial learning. * $p < 0.05$, *** $p < 0.001$ with respect to DMSO vehicle group.

PROJECT #2: ROLE OF PARP IN SECONDARY NEURONAL INJURY

PARP is a DNA repair enzyme whose activation requires considerable energy utilization. For this reason, it has been suggested that the activation of PARP in response to injury may actually contribute to necrotic cell death, by compromising the energy state of injured cells. Considerable work in cerebral ischemia has supported this concept. In contrast to cerebral ischemia, traumatic brain injury produces only modest changes in bioenergetic state except at very high injury levels. In our experiments we have examined the potential role of PARP in posttraumatic neuronal cell death using several model systems. In the first set of experiments, we obtained PARP knockout mice from DeMurcia's group in Europe. Breeding of these animals was done in our transgenic facility. PARP knockouts or a wild type controls were subjected to traumatic brain injury using our controlled cortical impact device. Although standard outcome variables in our laboratory include ability to walk a narrow beam and spatial learning/memory using the Morris Water Maze, only the latter could be utilized in our studies because PARP knockout mice even without injury were unable to walk the narrow beam. Surprisingly, PARP knockout animals did not differ from controls in a meaningful way. Although PARP knockout animals showed slightly reduced latencies to reach the submerged platform in the Morris Water Maze by Day 4 of the training trial, tests of working memory revealed the opposite trend (Fig. 3). In separate experiments, we used our rat lateral fluid percussion head injury model to examine the role of the somewhat selective PARP inhibitor (3AB). Similar to the mouse studies, inhibition of PARP had no significant effect on either motor or cognitive recovery following trauma (Fig. 4). Together, these studies suggest that activation of PARP following traumatic brain injury, in contrast to ischemic brain injury, does not appear to be a significant factor in posttraumatic cell death.

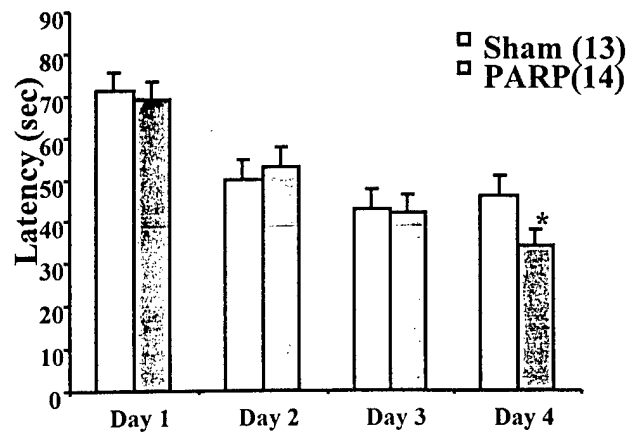


Figure 3. Morris water maze test following controlled cortical injury. PARP knockout mice showed improved spatial learning ability only on the last testing day. * $p < 0.05$ when compared with injured wild type mice.

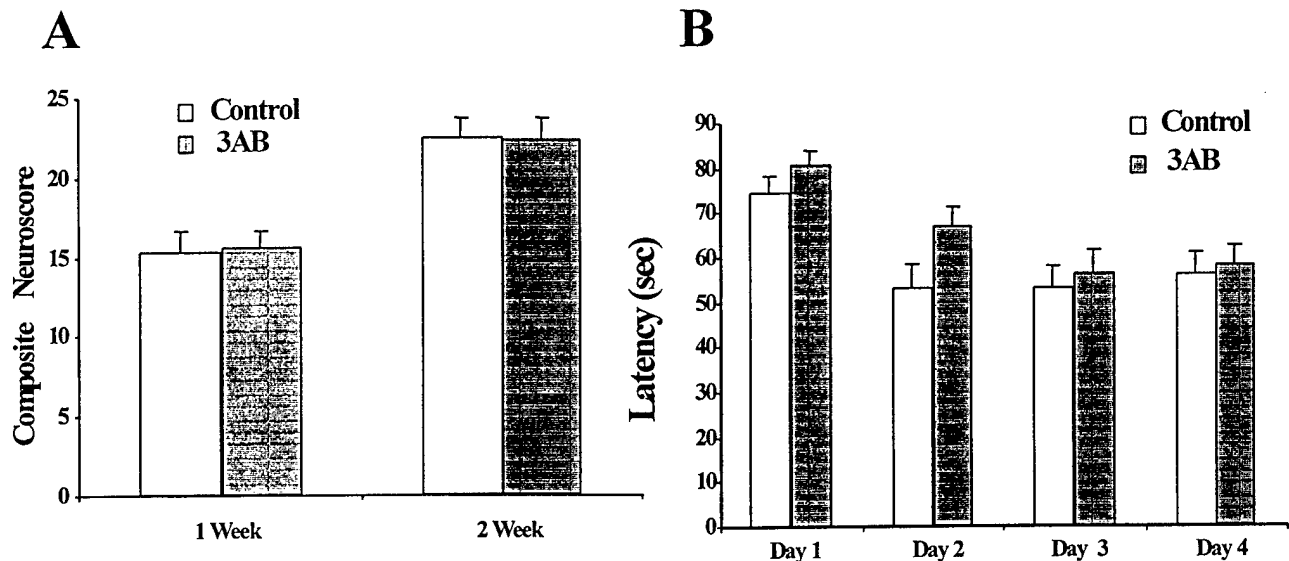


Figure 4. Recovery of neurological and cognitive function following fluid percussion injury in 3AB (PARP inhibitor) or vehicle icv treated rats. At two weeks post-trauma, 3AB treated rats did not show differences in motor function (A) or spatial learning ability in Morris water maze (B) as compared to vehicle treated controls.

PROJECT #3: ROLE OF CERAMIDE IN NEURONAL APOPTOSIS

Ceramide is a lipid produced through the actions of the enzyme sphingomyelinase, which has been implicated in both non-neuronal and more recently neuronal apoptosis. Our studies sought to confirm earlier results relating to the ability of a synthetic ceramide analog to produce neuronal apoptosis *in vitro*, as well as to examine whether endogenous ceramide produced such damage and was upregulated by apoptotic stimuli. Using both CGCs and cortical neurons grown in culture, we showed that the C_2 ceramide analog induced neuronal apoptosis in a dose dependent manner (Fig. 5). Parallel studies showed that administration of sphingomyelinase itself produced similar effects (Fig. 5). In addition, we produced apoptosis in CGCs by removal of serum and potassium; this caused apoptotic

cell death associated with increased levels of ceramide. Together, these findings indicate a potentially important role for ceramide in neuronal apoptosis.

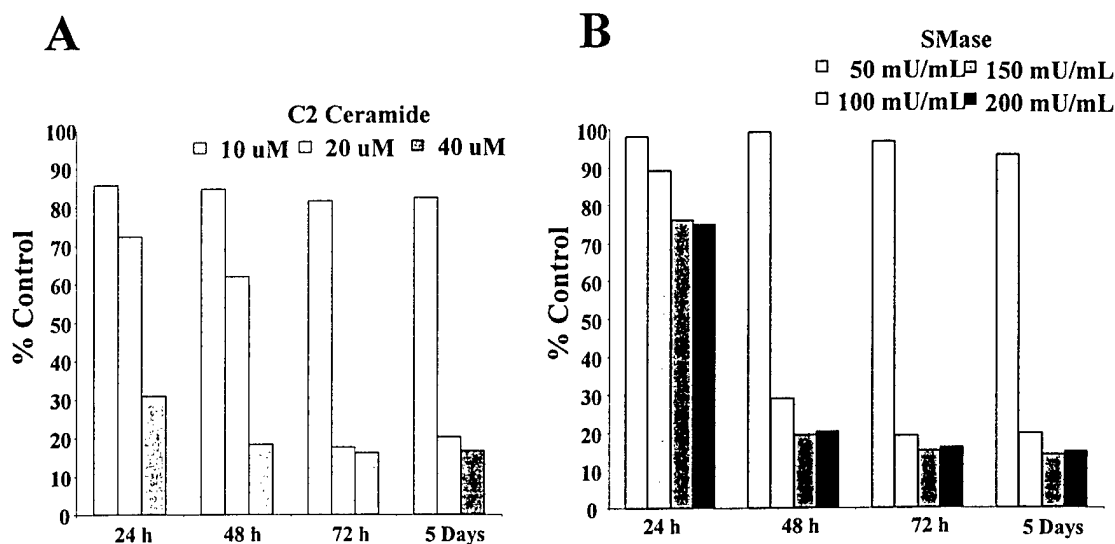
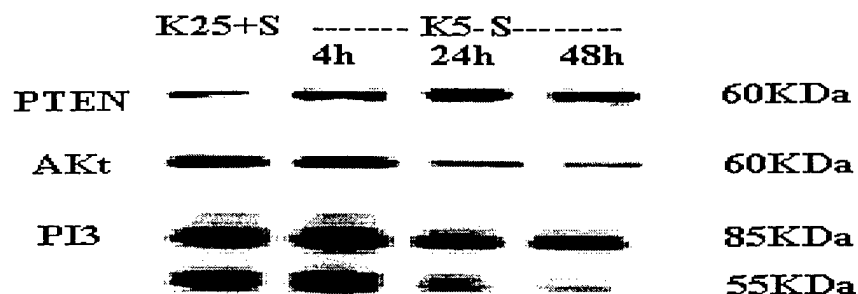


Figure 5. Levels of cell death in 7 DIV serum deprived CGC cultures (expressed as percent of control uninjured cultures) following the exogenous application of C2 Ceramide (A) or Sphingomyelinase (SMase) (B). Both C2 Ceramide and SMase are show dose and time dependent cell death.

PROJECT #4: ROLE OF PTEN IN NEURONAL APOPTOSIS

PTEN is a tumor suppressor gene, mutations of which have been implicated in many types of human cancers. Based on parallels with the tumor suppressor gene, p53, we hypothesize that PTEN may be involved in neuronal apoptosis. We demonstrated that apoptosis of cerebellar granule cells subjected to trophic withdrawal is associated with a significant upregulation of PTEN mRNA and protein levels (Fig. 6). Moreover, transfection with PTEN and cerebellar granule cells itself caused apoptosis. In addition, transfection with antisense oligonucleotides developed against PTEN protected cells from neuronal apoptosis caused by trophic withdrawal, whereas transfection with sense did the converse (Fig. 7). Most recent studies suggest that the apoptotic effects of PTEN may be related to its ability to downregulate the anti-apoptotic protein Akt. These are the first studies to demonstrate a role for PTEN in neuronal apoptosis and open a relatively new investigational area.

A



B

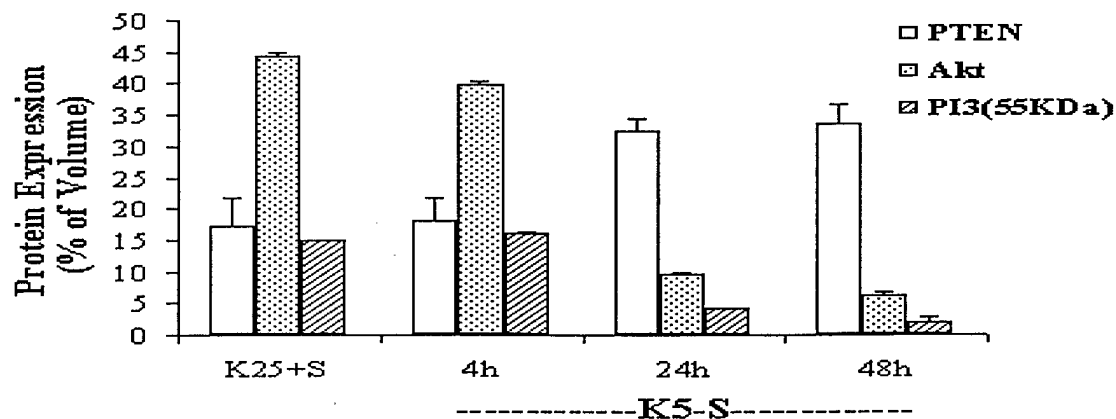


Figure 6A. Levels of protein expression for PTEN, Akt and PI3 as a function of time following serum/K⁺ deprivation of CGCs.

Figure 6B. Levels of PTEN are significantly increased following trophic withdrawal, whereas expression of Akt and PI3 (55KDa) are markedly decreased. Changes in protein level for PTEN and Akt are plotted in immun-BlotTM PVDF membrane (Bio-Rad, cat#162-0174). The bands of protein are quantitatively assessed using Bio-Rad, Image Densitometer (Model GS-700) through Multi-analysis software system.

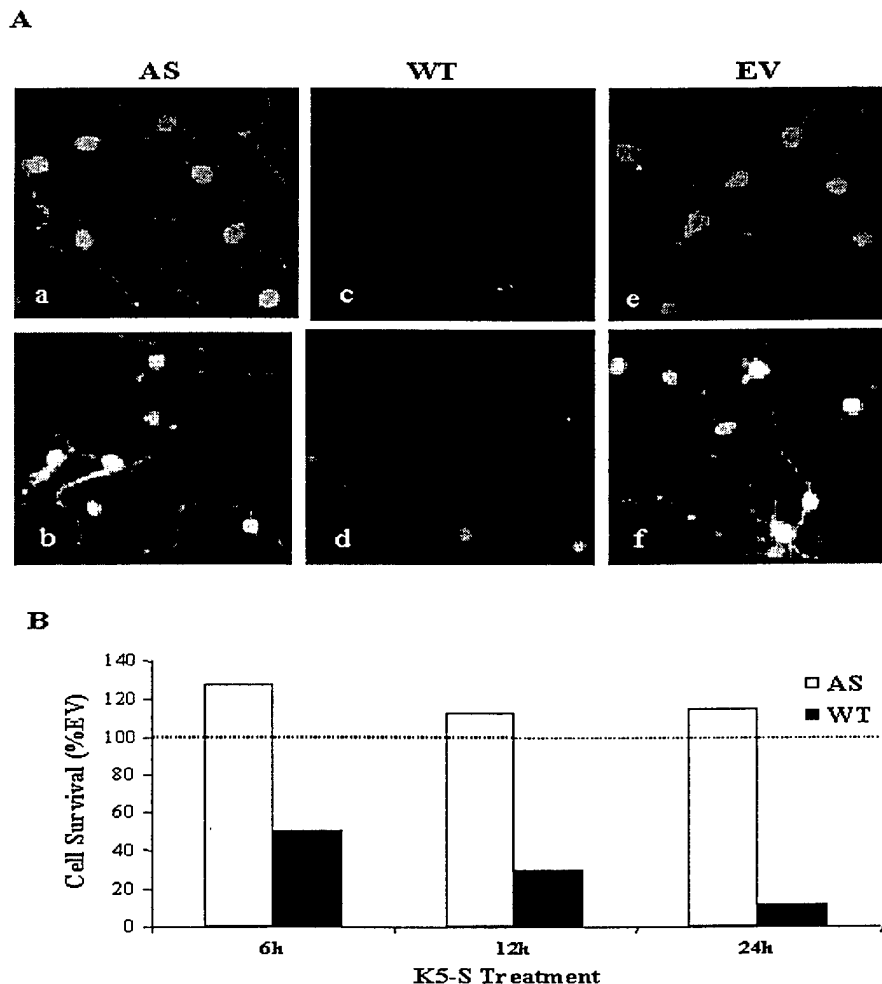


Figure 7A. Using a green fluorescent protein reporter, CGCs were transfected with plasmids containing antisense to PTEN (AS), wild type PTEN (WT), or empty vector (EV). The panels show morphological patterns of CGCs; transfection with WT caused apoptosis of CGCs (c), even in the presence of serum and high concentrations of potassium compared to AS (a) or EV (e). Using Hoechst 33258 staining after transfection following K⁺/serum deprivation at 6h, the panels show transfection with AS (b) reduces apoptosis compared to WT (d) or EV (f).

Figure 7B. Cell survival is plotted as a percentage change versus EV (100%). Transfection with WT PTEN causes cell death, whereas transfection with AS is protective. Histograms reflect percentage as compared to EV.

PROJECT #5: ROLE OF MGLUR IN SECONDARY CELL DEATH

We have previously established, in prior years of this grant, that metabotropic glutamate receptors play an important role in modulating posttraumatic neuronal cell death. Specifically, we demonstrated that antagonists to group I mGluR were neuroprotective following traumatic neuronal injury *in vitro* or following lateral fluid percussion injury to rats *in vivo*. In contrast, activation of groups II and III mGluR were neuroprotective. Recent studies have suggested that under certain conditions, however, activation of group I mGluR may provide protection to neurons (Fig. 8). We speculated that group I mGluR activation may have opposite effects on necrotic versus apoptotic stimuli. In our previous studies showing that activation of group I mGluR exacerbated injury and that antagonists were neuroprotective, the models shared largely necrotic profiles of cell death. Using mixed cortical glial cultures from rat cortex, we showed that in contrast to an exacerbating effect of group I mGluR on necrotic cell death, a Group I agonist had a clear neuroprotective effect to cells subjected to apoptotic stimuli using either staurosporine or etoposide (Fig. 8). Group I mGluR consists of two subclasses, mGluR1 and mGluR5. We have previously demonstrated that mGluR1, but not mGluR5, activation exacerbates traumatic neuronal cell death in a model that is largely necrotic. Therefore, we speculate that in contrast to mGluR1, that mGluR5 may provide protection under conditions of apoptosis.

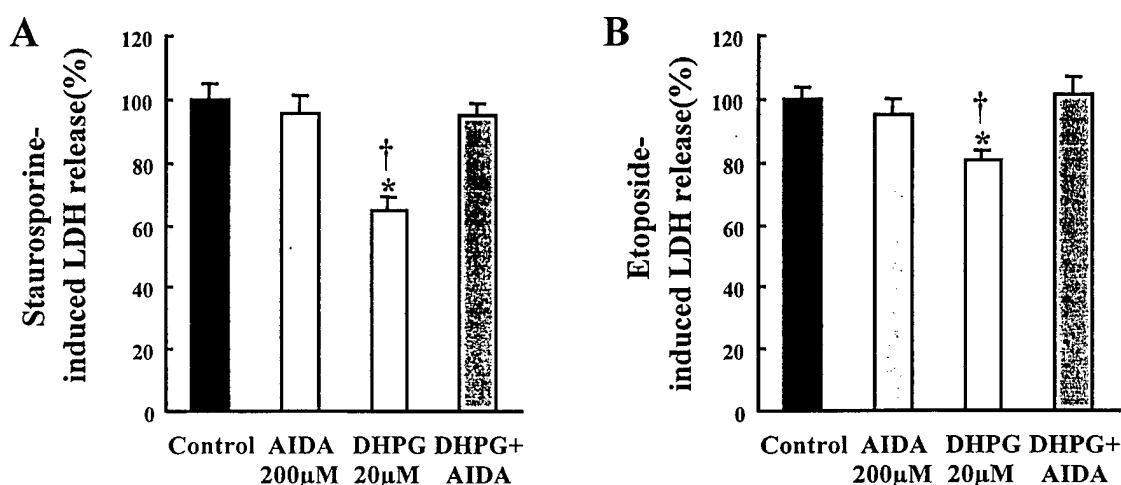


Figure 8. Activation of group I mGluR protects against staurosporine- and etoposide-induced cell death. (A) Application of the group I mGluR agonist DHPG significantly attenuated LDH release at 24 h induced by staurosporine (0.3 μM). In contrast, the group I mGluR antagonist AIDA was without effect on this injury but co-application of AIDA with DHPG completely reversed the protective effects of DHPG. (B) Administration of DHPG also significantly protected against cell death at 24 h induced by etoposide (2.5 μM). Analogous to the lack of effect of AIDA in staurosporine-induced injury, AIDA had no effect on this injury, but completely reversed the protective effects of DHPG when administered simultaneously. Combined treatment with DHPG and AIDA was performed at the same concentrations as single application. Bars represent mean \pm SEM, $n = 27$ -32 cultures per condition. Data expressed as a percentage of staurosporine- (A) or etoposide-induced (B) LDH release. * $p < 0.05$ vs. staurosporine (A) or etoposide (B) treatment; $^{\dagger}p < 0.05$ vs. AIDA + DHPG (ANOVA followed by Student-Newman-Keuls test).

PROJECT #6: DRUG DISCOVERY

In collaboration with Dr. Alan Kozikowski at our Institute, we have continued to develop novel small peptides – tripeptides and diketopiperazines – which show both neuroprotection and cognitive enhancing properties. The compounds were originally developed from a structure related to naturally occurring hormone, thyrotropin releasing hormone (TRH). However, our compounds have been modified to exclude all the known major physiological effects of TRH – endocrine, autonomic, analeptic – while actually enhancing the neuroprotective and cognitive enhancing effects of this compound. For the past year we have extensively studied these compounds in both *in vivo* and *in vitro* model systems and also have designed second generation compounds with sidegroups that target additional specific proposed injury factors such as glutamate or free radicals (Fig. 9,10).

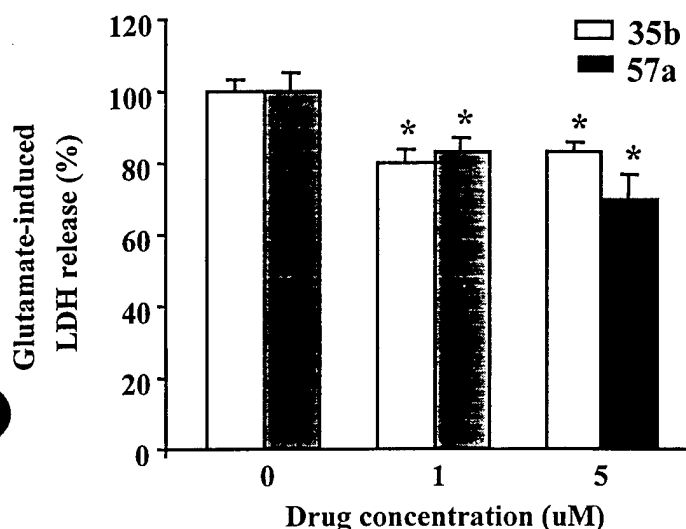


Figure 9: Treatment with 35b or 57a attenuated cell death induced by glutamate, suggesting that one potential action of these drugs may involve protection from excitotoxic mechanisms of secondary injury. Mixed rat neuronal-glial co-cultures were treated with 35b or 57a 30 min. before, during, and for 16-18 hr after incubation with 500uM glutamate. LDH activity was measured 16-18 hr after injury. Bars represent the mean±SEM for *n*=24 wells/condition. **p*<0.01 vs. control (injured, untreated) using *t*-test with Bonferroni correction.

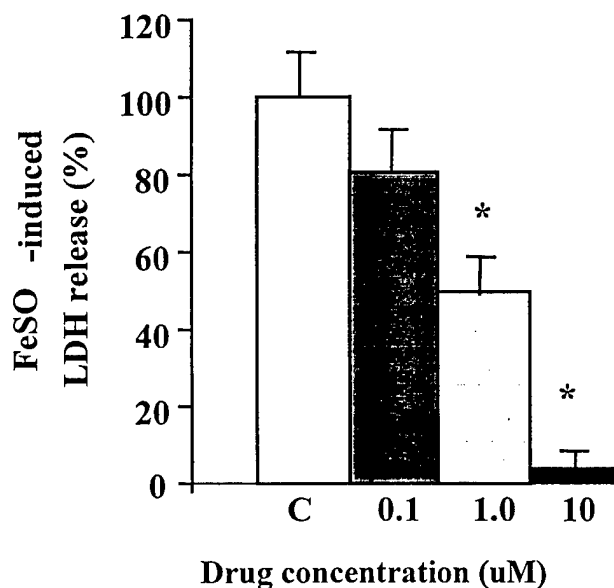


Figure 10: Treatment with 606mp dose-dependently increased survival in a FeSO₄ model of free radical-induced cell death. Neuronal-glial co-cultures were incubated with 100 uM FeSO₄ in the presence or absence of 606mp (0.1-10 uM). LDH activity was measured in an aliquot of media 16-18 hr later. Data are expressed as the percentage of injury-induced LDH activity seen in injured, untreated cultures (bar labeled C). Bars represent the mean±SEM for *n*=21 wells/condition. **p*<0.001 vs. FeSO₄ only (injured, untreated), using *t*-tests with Bonferroni correction.

Several of these compounds show remarkable effects, both *in vivo* and *in vitro*, including an ability to enhance both motor and cognitive recovery. The most recent studies demonstrate that these compounds have a remarkably long therapeutic window of at least 4 h (Fig. 11), and that they show a relatively flat, inverted U-shaped dose response curve. At the present time, studies are underway to examine potential mechanisms of action. Early work suggests that these peptides have multipotential actions and can modulate a number of proposed injury factors, including those involved in both necrotic cell death (Fig. 12) and in apoptotic cell death (Fig. 13).

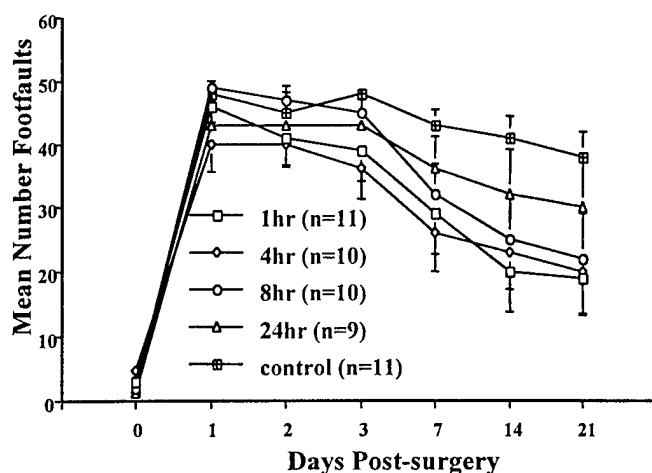


Figure 11. The time course of 35b administered at 1, 4, 8 and 24 h after controlled cortical impact injury versus vehicle control animals. The footfault is expressed as Mean \pm SE. Significant differences were seen in 1 and 4 h treated groups when compared with control animals, with trends in favor of 8 h treatment.

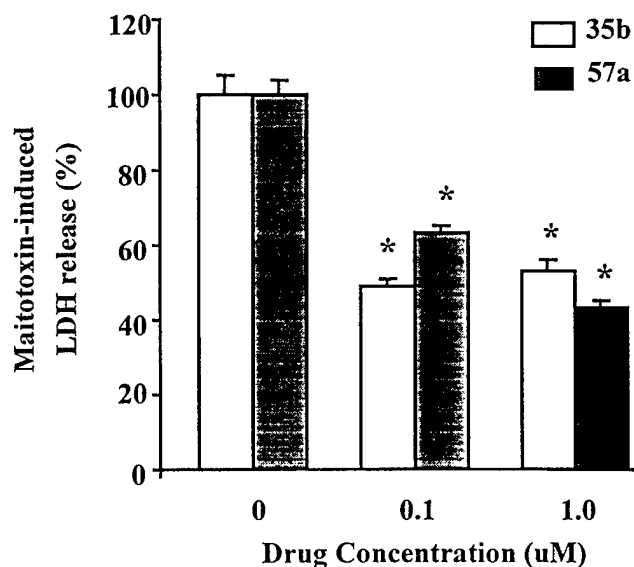


Figure 12: Treatment with 35b or 57a attenuated cell death in an *in vitro* model of necrotic injury. Mixed rat neuronal-glial co-cultures were treated with drugs during and after (16-18 hr) incubation with maitotoxin (0.1 nM for 1 hr). Maitotoxin induces necrotic death through rapid influx of calcium. LDH activity was assessed 16-18 hr after injury. Bars represent the mean \pm SEM for $n=24$ wells/condition. * $p<0.001$ vs. control (injured, untreated) using *t*-tests with Bonferroni correction.

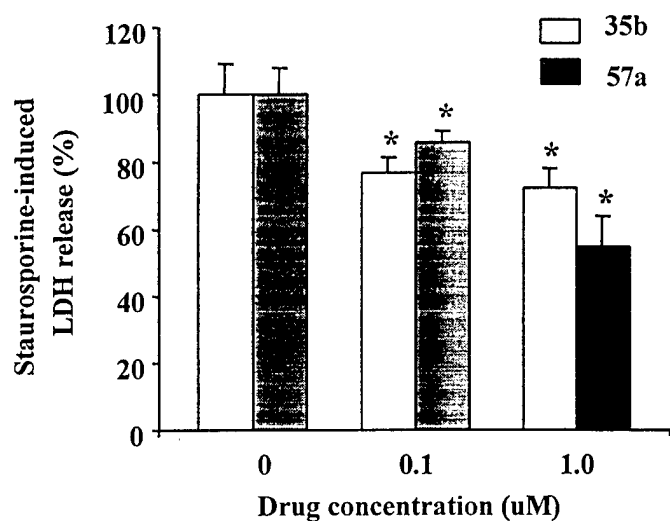


Figure 13: Treatment with 35b or 57a attenuated apoptotic cell death induced by staurosporine. Mixed rat neuronal-glial co-cultures were treated with drugs in the presence of staurosporine (0.1 uM) for 16-18 hr. LDH activity was assessed at the end of the incubation period. Bars represent the mean \pm SEM for $n=24$ wells/condition. Additional preliminary data (not shown) indicate that these drugs also reduce the number of cells with condensed or fragmented nuclei visualized with Hoechst 33258. * $p<0.05$ vs. control (injured, untreated) using t -test with Bonferroni correction.

SHERIDAN L. SWOPE, Ph.D.

Introduction

Neurotransmitter receptors are of primary importance in synaptic transmission and are targets for regulation. Modulation of the function, expression, or density of receptors has a profound effect on synaptic efficacy and thus may mediate plasticity. Accumulating evidence supports a role for phosphorylation in the regulation of synaptic transmission (Swope et al., 1992; Swope et al., 1999). Protein tyrosine kinases are a unique class of kinases initially found to mediate cell transformation and proliferation. However, protein tyrosine kinases are also involved in differentiated cell function. In fact, many protein tyrosine kinases are most highly expressed in the brain, being present both pre- and postsynaptically, suggesting their importance in the regulation of synaptic activity. Our long-term goal is to study the role of protein tyrosine kinases in synapse formation and function.

Much of the current understanding of synapses originates from studies of the neuromuscular junction (NMJ). The nicotinic acetylcholine receptor (AChR) is the ligand gated ion channel that mediates rapid postsynaptic depolarization at the NMJ. Because of its abundance in the electric organs of *Torpedo californica*, the AChR is the best characterized neurotransmitter receptor and has served as a model to elucidate the structure, function, and modulation of neurotransmitter receptors and ion channels. The AChR is phosphorylated on tyrosine residues both *in vitro* and *in vivo*, and this tyrosine phosphorylation is correlated with a modulation of the rate of receptor desensitization. In addition, tyrosine phosphorylation of the AChR and/or other postsynaptic components is involved in the nerve induced clustering of the AChR during synaptogenesis at the NMJ. Furthermore, protein tyrosine phosphorylation mediates the effect of acetylcholine receptor inducing activity (ARIA) to increase AChR transcription by nuclei underlying the synapse. Our interest has been to identify protein tyrosine kinases that are expressed postsynaptically at the NMJ which function to regulate the AChR.

We have identified two Src like kinases, Fyn and Fyk, that together comprise the predominant protein tyrosine kinase activity in the AChR enriched postsynaptic membrane of *Torpedo* electric organ (Swope and Haganir, 1993). Src class kinases are also present in skeletal muscle and brain. Fyn and Fyk associate with the AChR via a binding of their src homology 2 (SH2) domains to the tyrosine phosphorylated δ subunit of the receptor (Swope and Haganir, 1994). In addition, Fyn and Fyk phosphorylate the receptor *in vitro* (Swope and Haganir, 1993). Other laboratories have demonstrated that Src itself also associates with the AChR of muscle. These initial studies suggest that Src family kinases are important regulators of synaptic function at the NMJ and in the central nervous system.

Laboratory Development

Over the last year, Dr. Ali Mohamed, a postdoctoral fellow with biochemical and molecular biological experience in the area of protein phosphorylation and Dr. William Rosoff, a postdoctoral fellow with cell biological experience in the area of synapse formation have continued to be members of the laboratory. In addition, Ms. Anne Miermont, a research assistant, has been with the laboratory for 18 months. The efforts of these researchers will enable our laboratory to attain its research goals. Extramural funding from the National Institutes of Health, National Institute of Neurological Disorders

and Stroke in the form of an R29 or First Award has been successfully extended via a noncompetitive renewal. Furthermore, funding through the Muscular Dystrophy Association has also been renewed.

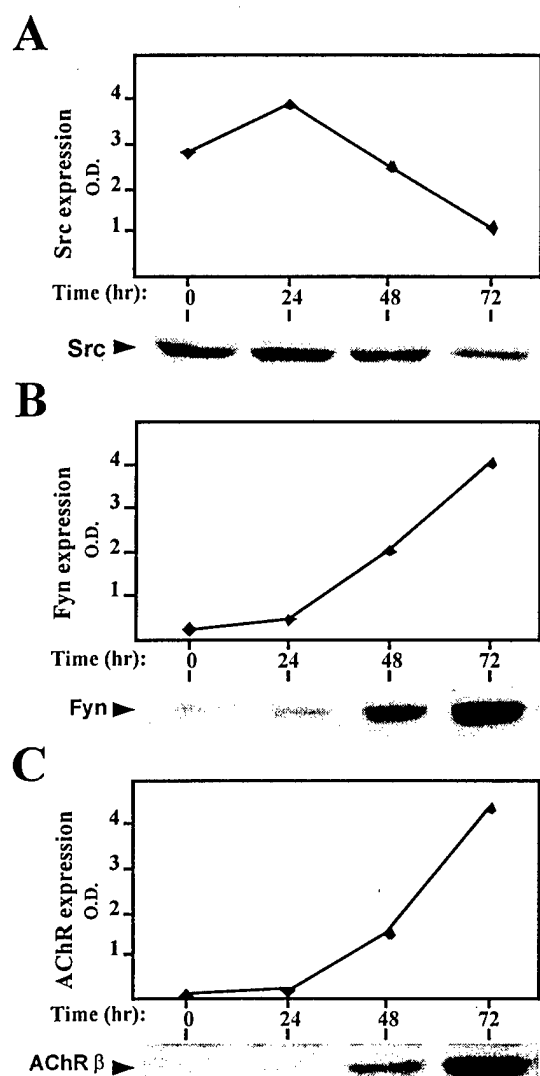


Fig 1. Expression of Src class kinases during muscle cell differentiation. C2 myoblasts were cultured to 80% confluence and switched to low serum at $t=0$. After 0, 24, 48, or 72 hrs, cells were harvested with 2% SDS Lysis Buffer. Cellular lysates representing 20 μ g of protein were resolved by SDS PAGE and analyzed by immunoblotting for (A) Src, (B) Fyn, and (C) AChR β subunit. In each of A, B, and C, the lower panel represents Western analysis and the position of Src, Fyn, and the AChR β subunit, respectively, are indicated by an arrow. The upper panels in A, B, and C represents quantification of the Western analysis by densitometry scanning.

As an established laboratory at the Georgetown Institute of Cognitive and Computation Sciences, the aim of our current research is to continue to test the hypothesis that Src class kinases are involved in synapse formation and function at the NMJ by regulating the AChR. Furthermore, the molecular mechanisms by which these kinases act to regulate synaptic transmission are being investigated.

Clarification of the Mechanisms for Regulation of Src class Kinases at the NMJ

One of our research goals is to identify mechanisms by which Src class kinases are regulated at the NMJ. Prior to innervation, embryonic myoblasts fuse into myotubes. This differentiation process results in an upregulation of AChR expression. Our recent results have demonstrated a specific coregulation of Fyn (Fig 1). During fusion of the C2C12 mouse muscle cells, Fyn was upregulated in concert with the AChR. In contrast, expression of Src was unchanged during differentiation. This specific coregulation of Fyn with the AChR supports the importance of Fyn at the NMJ. This study has been submitted for publication (Rosoff and Swope, 1999).

Investigation of the primary extracellular factors that regulate the activity of Src kinases was also initiated. Agrin is the neuron-derived factor that induces AChR phosphorylation and clustering. Our preliminary results indicate that the agrin receptor, MuSK, may be important for regulating Src kinases. Direct *in vitro* binding between MuSK and Src kinases was demonstrated using fusion protein affinity chromatography (data not shown). In addition, complex formation in muscle cells between MuSK and Src or Fyn was activated by agrin (Fig 2). These results suggested that agrin, acting via MuSK, activates Src class kinases. These data are in preparation for publication.

Clarification of the Signal Transduction Cascade Involving Src class kinases

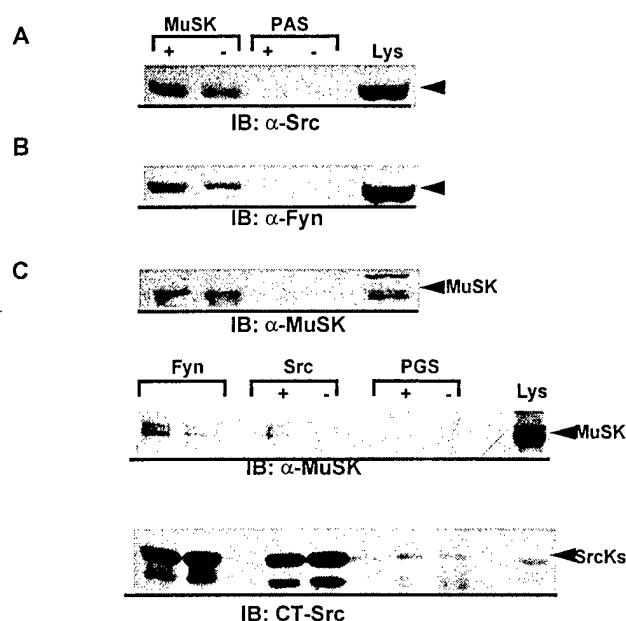


Fig 2. Effect of Agrin on co-immunoprecipitation of MuSK with Fyn and Src. (A) C2 myotubes were treated with 100 pM agrin (+) or an equal volume of conditioned medium (-) for 1 hour. Myotubes were solubilized in 1% Triton Lysis Buffer, centrifuged for 10 min at 225,000 x g, and the supernatants immunoprecipitated with a mixture of goat polyclonal anti-MuSK antibodies, N19 and C19 (MuSK) or empty protein A-sepharose (PAS). The immunoprecipitates and 100 µg of lysates supernatant after centrifugation (Lys) were resolved by 8% SDS-PAGE, and analyzed by Western blotting using Src specific mouse monoclonal antibody, GD11 (α-Src). Arrow indicates the position of Src. (B) The blot from A was stripped and reanalyzed by Western blotting using Fyn specific rabbit polyclonal antibody, Fyn3 (α-Fyn). Arrow indicates the position of the Fyn. (C) The blot from A was stripped and reanalyzed by Western blotting using MuSK specific rabbit polyclonal antibody (α-MuSK). Arrow indicates the position of the MuSK. (D) C2 myotubes were treated with agrin or conditioned medium and solubilized as in A. The supernatants after centrifugation were immunoprecipitated with Fyn specific mouse monoclonal antibody, Fyn15 (Fyn), or Src specific mouse monoclonal antibody, Ab-1 (Src) or empty protein G-sepharose beads (PGS). The immunoprecipitates and 100 µg of lysates supernatant after centrifugation (Lys) were resolved by 7% SDS-PAGE, and analyzed by Western blotting using MuSK specific rabbit polyclonal antibody (α-MuSK). Arrow indicates the position of MuSK. (E) The blot from D was stripped and reanalyzed by Western blotting using Src family kinase specific rabbit polyclonal antibody, Src2 (CT-Src). Arrow indicates the position of Src kinases.

We are also in the process of characterizing the signal transduction pathway(s) by which Src class kinases act to regulate synapse formation and function by asking: what are the identities of cellular components that interact directly with Fyn, Fyk, and Src? These ongoing studies will be performed using coimmunoprecipitation techniques, fusion protein affinity chromatography, and the yeast two-hybrid system. These methods have become generally applicable for the identification of target proteins based on protein-protein interaction including protein kinase substrates.

Using the yeast two-hybrid system, we identified a molecular component of *Torpedo* electric organ that interacts with the unique domain of Fyn. This 13kDa component was homologous to a previously identified mouse sterility factor, Tctex-1 that is a light chain of the cytoplasmic microtubule protein dynein (Mou et al., 1998). We tested whether *Torpedo* Tctex-1 was complexed with dynein or cytoskeletal elements of the NMJ including rapsyn and syntrophin. Precipitation of Tctex-1 resulted in specific coprecipitation of the intermediate chain, IC74, of dynein. Further evidence for Tctex-1 being a component of dynein in *Torpedo* electric organ was obtained by immunoprecipitating dynein IC74 and analyzing the precipitate for Tctex-1 by Western blotting. Tctex-1 was specifically coprecipitated with dynein. These indicated that Tctex-1 was a component of the molecular motor protein dynein in *Torpedo* electric organ.

Association of Src class kinases with the Tctex/dynein complex was also tested. The presently available anti-Fyn antibodies were not useful for directly demonstrating association of Fyn with the Tctex/dynein complex. However, in four experiments, precipitation of dynein from electric organ homogenate resulted in

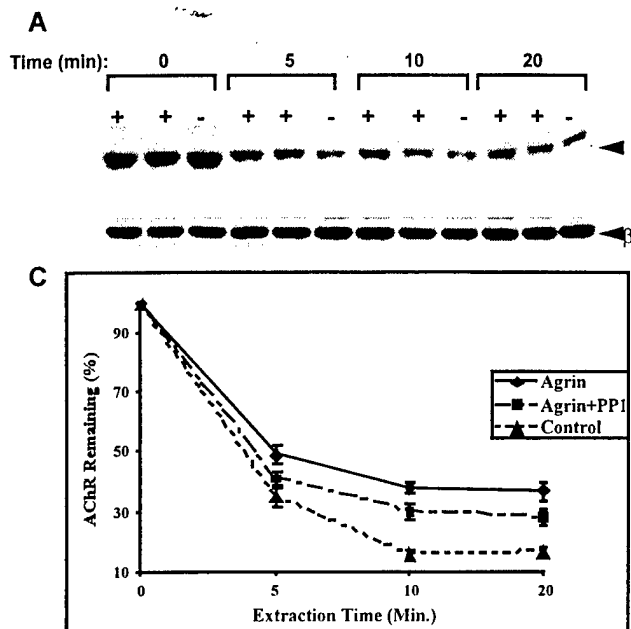


Fig 3. Effect of Inhibiting Src-class Kinases on Agrin-induced AChR Cytoskeletal Anchoring (A) C2 myotubes were treated with 10-20 μ M PP1 (PP1) or DMSO vehicle for 1h followed by treatment with 100 pM agrin (Agrin) or conditioned medium (Control) for 1h. Treated myotubes were rinsed with ice cold PBS, extracted on ice with Prives buffer containing 0.5% triton X-100 for the indicated times. Plates were rinsed once with Prives buffer containing 0.1% Triton X-100. The remaining proteins were extracted using SDS-PAGE sample buffer, sonicated for 30s, and centrifuged for 10 min at 225,000 x g. Aliquots of the extracted proteins representing 10% of 35 mm dish were resolved by 8% SDS-PAGE, and analyzed by Western blotting using a rat monoclonal antibody #148 specific of the AChR β subunit. (B) Sister C2 cultures were treated with or without agrin, with or without PP1, extracted with Prives buffer without Triton X-100. Plates were rinsed once with Prives buffer without Triton X-100. The remaining proteins were extracted using SDS-PAGE sample buffer, sonicated for 30s, and centrifuged for 10 min at 225,000 x g. Aliquots of the extracted proteins representing 10% of 35 mm dish were resolved by 8% SDS-PAGE, and analyzed by Western blotting using a rat monoclonal antibody #148 specific of the AChR β subunit. (C) AChR immunoblots from 4 different experiments as in A were quantified by densitometric scanning of non-saturated pixles of films. The represent % of AChR remaining on the dish after 0.5% Triton X-100 extraction.

coprecipitation of a Src family kinase as demonstrated by Western analysis with an antibody to the carboxy terminal sequence of Src family kinases. Library screening as well as PCR analysis have identified Fyn and Fyk as the only Src class kinases in *Torpedo* electric organ. Thus, these data suggested that the Src class kinase associated with the Tctex/dynein complex was Fyn and/or Fyk.

The primary function of the endplate at the NMJ is to mediate synaptic transmission via the activation of the AChR. Since Tctex-1 was detected in postsynaptic membranes, complex formation between the cytoskeletal protein and the AChR was examined. Immunoprecipitation of Tctex-1 resulted in coprecipitation of the AChR indicating that the AChR was also contained in a complex with Tctex-1.

The sequence of *Torpedo* Tctex indicated a putative tyrosine phosphorylation site at Tyr4 (Mou et al., 1998). In fact, a Tctex fusion protein could be phosphorylated on tyrosine residue(s) *in vitro* by immunoprecipitated Fyn (Mou et al., 1998). These results suggested that Tctex may be a substrate for Fyn at the NMJ. Anchoring of postsynaptic components at the NMJ by cytoskeletal elements is now believed to involve tyrosine phosphorylation of postsynaptic components. Thus, Fyn may regulate AChR aggregation via an effect on the Tctex cytoskeletal element. These data have been published (Mou et al., 1998).

Determination of the Physiological Roles of Src class kinases

To determine the physiological role of Src class kinases in synapse formation and function, a strategy involving expression of recombinant kinases in a heterologous cell line that expresses the AChR has been developed. Coexpression of Fyn, Fyk, or Src and the endplate specific cytoskeletal protein rapsyn in the Q-F18 cells increased cellular tyrosine phosphorylation (Data not shown). The increase in cellular

phosphorylation occurred primarily in the cytoskeletal fraction (Data not shown). These data indicated that rapsyn was able to activate Fyn, Fyk, and Src.

Coexpression of Fyn, Fyk, or Src and rapsyn in the Q-F18 cells also increased phosphorylation of the AChR as demonstrated by phosphorylation state specific antibodies (Mohamed and Swope, 1999). AChR purified from Q-F18 cells transfected with the kinases and rapsyn were analyzed in parallel for AChR content and AChR tyrosine phosphorylation (Mohamed and Swope, 1999). Depending on the specific subunit and kinase analyzed, the effect of kinase plus rapsyn was 2-20 fold greater than the effect of rapsyn alone. These results indicated that all three kinases were capable of phosphorylating the AChR. In addition, Fyn-, Fyk-, and Src-mediated AChR phosphorylation occurred only upon expression of rapsyn. These data suggested that the ability of rapsyn to regulate Fyn, Fyk, and Src resulted in phosphorylation of the AChR by these kinases (Mohamed and Swope, 1999).

The physiological relevance of AChR phosphorylation by Src family kinases was examined by testing whether the phosphorylation was associated with anchoring of the receptor to the cytoskeleton. Little or no AChR was associated with the cytoskeleton in control cells or cells expressing Fyn, Fyk, or Src alone as demonstrated by Western analysis using antibodies to the AChR α , γ , and δ subunits (Mohamed and Swope, 1999). However, when Fyn, Fyk, and Src were expressed with rapsyn, there was a dramatic increase in the AChR found in the cytoskeletal fraction (Fig 6A). AChR was also detected at the cytoskeleton upon expression of rapsyn alone. However, activation of the coexpressed kinases by rapsyn resulted in greater translocation of the receptor to the cytoskeleton compared to rapsyn alone. Thus, for Fyn, Fyk, or Src plus rapsyn the increase in AChR at the cytoskeleton over the effect of rapsyn alone was 1.9 ± 0.1 , 1.7 ± 0.1 , and 2.6 ± 0.2 (mean \pm S.E.M.; $n=8$) respectively as determined by quantitative scanning densitometry. These data demonstrated that Fyn, Fyk, and Src could induce anchoring of the AChR to the cytoskeleton in a rapsyn dependent manner. In addition, the rapsyn stimulated effect of Src kinases to increase cytoskeletal anchoring of the receptor was essentially abolished by herbimycin A suggesting that kinase activity was necessary for AChR translocation (Mohamed and Swope, 1999). These data, which indicate an important role for Src kinases in AChR anchoring to the cytoskeleton have been published.

Src kinases also regulate cytoskeletal anchoring of the AChR in muscle cells. Treatment with agrin induces translocation of the AChR to the cytoskeleton and this effect of agrin is blocked by a highly specific Src kinase inhibitor (Fig 3). These data, which argue that Src kinases mediate the regulation of the AChR by agrin are in preparation for publication.

In summary we have made significant progress in demonstrating that Src class protein tyrosine kinases are involved in the regulation of the AChR of the NMJ. Clarification of these basic molecular processes are relevant to an understanding of synaptic transmission in healthy people as well as those afflicted with neurological and neuromuscular diseases.

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